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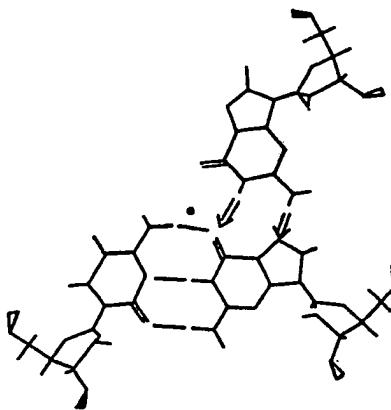
WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : <b>C07H 15/12, A22C 9/00 C12N 15/00, A61K 48/00</b>	A1	(11) International Publication Number: <b>WO 90/06934</b> (43) International Publication Date: <b>28 June 1990 (28.06.90)</b>
(21) International Application Number: <b>PCT/US89/05769</b>		(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).
(22) International Filing Date: <b>20 December 1989 (20.12.89)</b>		
(30) Priority data: <b>287,359 20 December 1988 (20.12.88) US</b>		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
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(54) Title: TRIPLE STRANDED NUCLEIC ACID AND METHODS OF USE



(57) Abstract

A method for making synthetic oligonucleotides which bind to target sequences in a duplex DNA forming colinear triplexes by binding to the major groove. The method includes scanning genomic duplex DNA and identifying nucleotide target sequences of greater than about 20 nucleotides having either about at least 65% purine bases or about at least 65% pyrimidine bases; and synthesizing synthetic oligonucleotides complementary to identified target sequences. The synthetic oligonucleotides have a G when the complementary location in the DNA duplex has a GC base pair and have a T when the complementary location in the DNA duplex has an AT base pair. The synthetic oligonucleotides are oriented 5' to 3' and bind parallel or 3' to 5' and bind anti-parallel to the about at least 65% purine strand. Also described are synthetic oligonucleotides made by the above methods. The oligonucleotides can be altered by modifying and/or changing the bases, adding linkers and modifying groups to the 5' and/or 3' termini, and changing the backbone. These synthetic oligonucleotides bind to duplex DNA to form triplexes. This process alters the functioning of the genes which are bound. This process can be used to inhibit cell growth, alter protein ratios, treat diseases including cancer and permanently alter the DNA.

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Triple Stranded Nucleic Acid and Methods of Use

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This application is a Continuation-in-Part of  
Applicants Co-pending U.S. Application Serial No. 287,359,  
20 filed December 20, 1988.

This invention was supported in part through a  
grant or award from the National Institute of Health.

25

FIELD OF INVENTION

The present invention relates generally to a  
method for making synthetic oligonucleotides which bind to  
the major groove of a duplex DNA to form a colinear  
triplex. It also relates to synthetic oligonucleotides  
30 which bind to the purine strand of a DNA duplex. It

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1 further relates to a method of regulating and inhibiting  
cellular growth by administering a synthetic  
oligonucleotide which is capable of binding to a DNA  
duplex to form a colinear triplex.

5

BACKGROUND ON THE INVENTION

It has been known for some time that the polynucleotide polydT will bind to the polydA-polydT duplex to form a colinear triplex (Arnott, S & Selsing E. (1974) J. Molec. Biol. 88, 509). The structure of that triplex has been deduced from X-ray fiber diffraction analysis and has been determined to be a colinear triplex (Arnott, S & Selsing E. (1974) J. Molec. Biol. 88, 509 ).  
10 The polydT strand is bound in the parallel orientation to the polydA strand of the underlying duplex. The polydT-polydA-polydT triplex is stabilized by T-A Hoogstein base pairing between A in the duplex and the third strand of polydT. That interaction necessarily  
15 places the third strand, called a ligand, within the major groove of the underlying duplex. The binding site in the major groove is also referred to as the target sequence.  
20

Similarly, it has been shown that polydG will bind by triplex formation to the duplex polydG-polydC, presumably by G-G pairing in the major helix groove of the underlying duplex, (Riley M., Mailing B. & Chamberlin M. (1966) J. Molec. Biol. 20, 359). This pattern of association is likely to be similar to the pattern of  
25 G-G-C triplet formation seen in tRNA crystals (Cantor C. & Schimmel P., (1980) Biophysical Chemistry vol I, p. 192-195).

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1           Triplices of the form polydA-polydA-polydT and  
polydC-polydG-polydC have also been detected (Broitman S.,  
Im D.D. & Fresco J.R. (1987) Proc. Nat. Acad. Sci USA 84,  
5120 and Lee J.S., Johnson D.A. & Morgan A.R. (1979) Nucl.  
Acids Res. 6, 3073). Further the mixed triplex  
5           polydCT-polydGA-polydCT has also been observed. (Parseuth  
D. et al. (1988) Proc. Nat. Acad Sci. USA 85, 1849 and  
Moser H.E. & Dervan P.B. (1987) Science 238, 645). These  
complexes, however, have proven to be weak or to occur  
10          only at acid pH.

Parallel deoxyribo oligonucleotide isomers which  
bind in the parallel orientation have been synthesized  
(Moser H.E. & Dervan P.E. (1987) Science 238, 645-650 and  
15          Rajagopol P. & Feigon J. (1989) Nature 339, 637-640). In  
examples where the binding site was symmetric and could  
have formed either the parallel or antiparallel triplex  
(oligodT binding to an oligoA-oligodT duplex target), the  
resulting triplex formed in the parallel orientation  
20          (Moser H.E. & Dervan P.E. (1987) Science 238, 645-650 and  
Parseuth D. et al. (1988) PNAS 85, 1349-1353), as had been  
deduced from x-ray diffraction analysis of the  
polydT-polydA-polydT triplex.

25          Studies employing oligonucleotides comprising the  
unnatural alpha anomer of the nucleotide subunit, have  
shown that an antiparallel triplex can form (Parseuth D.  
et al. (1988) PNAS 85, 1349-1353). However, since the  
alpha deoxyribonucleotide units of DNA are inherently  
30          reversed with respect to the natural beta subunits, an  
antiparallel triplex formed by alpha oligonucleotides  
necessarily follows from the observation of parallel  
triplex formation by the natural beta oligonucleotides.  
For example, alpha deoxyribo oligonucleotides form

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1 parallel rather than antiparallel Watson-Crick helices  
with a complementary strand of the beta DNA isomer.

5 It has been demonstrated that a DNA  
oligonucleotide could bind by triplex formation to a  
duplex DNA target in a gene control region; thereby  
repressing transcription initiation (Cooney M. et. al.  
(1988) Science 241, 456). This was an important  
observation since the duplex DNA target was not a simple  
10 repeating sequence.

15 The present invention provides a new method for  
designing synthetic oligonucleotides which will bind  
tightly and specifically to any duplex DNA target. When  
the target serves as a regulatory protein the method can  
be used to design synthetic oligonucleotides which can be  
used as a class of drug molecules to selectively  
manipulate the expression of individual genes.

20 **SUMMARY OF THE INVENTION**

The object of the present invention is a method  
for designing synthetic obliogonucleotides which bind to  
duplex DNA.

25 A further object of the present invention is a  
method for making synthetic obliogonucleotides which form  
triplexes with DNA.

30 An additional object to the present invention is  
a synthetic oligonucleotide which forms a colinear triplex  
with a target sequence in a duplex DNA.

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1       Another object to the present invention is a provision of a synthetic oligonucleotide which inhibits the growth of cells.

5       A further object of the present invention is a provision of a synthetic oligonucleotide which inhibits the growth of a pathogen.

10      An additional object of the present invention is a method for altering the structural protein content of epidermal tissue for the treatment of aging and blood clotting.

15      A further object of the present invention is a method of inhibiting gene expression by permanently altering the DNA sequence.

20      Thus, in accomplishing the foregoing objects, there is provided in accordance with one aspect of the present invention a method for making a synthetic oligonucleotide which binds to a target sequence in duplex DNA forming a colinear triplex by binding to the major groove, said method comprising the steps of: scanning genomic duplex DNA and identifying nucleotide target sequences of greater than about 20 nucleotides having either about at least 65% purine bases or about at least 65% pyrimidine bases; and synthesizing said synthetic oligonucleotide complementary to said identified target sequence, said synthetic oligonucleotide having a G when the complementary location in the DNA duplex has a GC base pair, having a T when the complementary location of the DNA duplex has an AT base pair. In specific embodiments the synthetic oligonucleotide can be selected from the group consisting of an oligonucleotide oriented 5' to 3'

1 and binding parallel to the about at least 65% purine strand, or an oligonucleotide oriented 3' to 5' and binding anti-parallel to the about at least 65% purine strand.

5 A further aspect of the present invention is the synthetic oligonucleotide for forming a colinear triplex with a target sequence in a duplex DNA when said target sequence is either about at least 65% purine bases or about at least 65% pyrimidine basis, comprising, a nucleotide sequence of at least about 20 nucleotides; said nucleotide sequence including G and T, wherein G is used when the complementary location and duplex DNA has a GC base pair and T is used when the complementary location in the duplex DNA is an AT base pair; and said sequence selected from the group consisting of an oligonucleotide oriented 5' to 3' and binding parallel to the about at least 65% purine strand of the duplex DNA target sequence, and an oligonucleotide oriented 3' to 5' and binding anti-parallel to the about at least 65% purine strand in the duplex DNA target sequence.

In the preferred embodiments the synthetic oligonucleotide can have at least one T replaced by X, I, and halogenated derivatives of X and I. Furthermore, at least one G can be replaced with halogenated derivatives of G.

Additional embodiments include substitutions on the synthetic oligonucleotide. For example, the base can be substituted at the 2' furanose position with a non-charged bulky group and the backbone of the synthetic oligonucleotide can be a phosphodiester analogue which is not readily hydrolyzed by cellular nucleases. In

1 addition, a linker can be affixed at the 3' and/or 5'  
terminus of the synthetic oligonucleotide. This linker  
provides a method for attaching modifying groups to the  
oligonucleotide. The modifying groups can be  
5 intercalators, groove-binding molecules, cationic amines  
and cationic polypeptides.

Another aspect of the present invention is a  
method of inhibiting the growth of cells comprising the  
10 step of administering synthetic oligonucleotides in  
sufficient quantity for cellular uptake and binding to the  
target sequence, wherein said target sequence is  
positioned within the DNA domain adjacent to the RNA  
transcription origin. This procedure can be used to  
15 inhibit the growth of cancer cells and pathogens. In one  
preferred embodiment this procedure is used to inhibit  
HIV-I virus by binding a synthetic oligonucleotide to the  
viral LTR region.

20 Another aspect of the present invention is a  
method of altering the relative proportions of the  
structural protein content of epidermal tissue by  
administering a synthetic oligonucleotide in sufficient  
quantity for cellular uptake and binding to target  
25 sequences for collagen genes.

Other and further objects, features and  
advantages will be apparent from the following description  
of the presently preferred embodiments of the invention  
30 given for the purpose of disclosure when taken in  
conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A shows the surface morphology of a colinear triplex. It is a computer generated rendering of the structure of a duplex DNA target site and presents in both the canonical B and A helix form. Upon binding of an oligonucleotide ligand, the target undergoes a transition from the B to the A form, which creates an increase in the depth of the major helix groove (M). In a colinear triplex, the oligonucleotide wraps about the A form helix target, occupying the major groove. The groove binding has been emphasized by presenting the bound oligonucleotide as a ribbon-like abstraction.

Fig. 1B shows the strand orientation in a colinear triplex. The oligonucleotide ligand binds to the duplex target, in the parallel orientation relative to the orienting (more purine rich) strand.

Fig 2 shows the pattern of oligonucleotide hydrogen bonding with the duplex target: G to GC sites, T to AT sites. 2A is a computer simulated rendering of the preferred pattern of hydrogen bonding between G in the ligand and G in the GC base pair at the corresponding site within the orienting strand of the duplex target. 2B is an equivalent simulation of T binding to the A of an AT base pair at its corresponding site within the orienting strand of the duplex target. The T-AT association is identical to classical "Hoogsteen base pairing", whereas the G-GC association is essentially the guanine counterpart thereof and involves N3 to O6 bonding. Solid wedges define the site at which such a crossection through a triplex is affixed to the corresponding crossection above it. Open wedges define the site at which such a

1 crossection through a triplex is affixed to the  
corresponding crossection below. As seen, the  
connectivity defined by the two bonding schemes is nearly  
identical. It is also important to recognize that the  
5 favored pattern of bond formation between G and GC or T  
and AT (arrows) cannot be mimicked by any other pattern of  
base-base association at neutral pH (C can mimic G in acid  
conditions).

10 2C and 2D are corresponding bonding patterns  
which result when the G of a GC base pair or A of an AT  
pair occurs across from the orienting strand of the target  
duplex. In that instance, the rules of oligonucleotide  
sequence selectivity are the same (i.e., G at a GC pair, T  
15 at an AT pair) however, G bonding occurs N3 to N9 and T  
bonds in the "reverse Hoogsteen" way, thereby both retain  
the overall parallel orientation of the bound ligand and  
the orienting strand of the target.

20 Fig. 3 shows one method of improving the pattern  
of oligonucleotide hydrogen bonding with the duplex  
target: xanthine binding to AT sites. The computer  
generated simulation in Fig. 3 is as in Fig. 2, except  
that the effect of substituting xanthine (X) for T is  
25 presented. As seen, in both the "Hoogsteen" binding (3A  
and 3B) and "Reverse Hoogsteen" (3C and 3D) mode of  
binding, X and T bind equivalently to an underlying AT  
base pair. The major difference between the two is that X  
is nearly identical to the G residues which might flank it  
30 in an oligonucleotide ligand, with respect to base size  
and shape and with respect to the orientation of its  
phosphodiester component within the oligonucleotide  
binding site. Modeling predicts that such enhancement of  
oligonucleotide continuity will enhance the binding  
35 affinity and site specificity of all oligonucleotides in  
which T is replaced by X.

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1 Fig. 4 displays the family of altered  
phosphodiester linkages compatible with colinear triplex  
formation. Some of the homologues of the phosphate within  
the backbone of an oligonucleotide are presented. In each  
5 instance, examples are cited which can be prepared by a  
simple modification of the standard computer assisted,  
solid phase methods. Examples A-C are thiophosphate  
linkage, E is methylphosphonate, F is phosphoramidite and  
G is phosphotriester.

10 Fig. 5 shows formation of hybrid oligonucleotides  
by means of coupling through a 5' amine linkage. In this  
instance, a hexylamine linkage is described. This linkage  
can be affixed as the last residue of an oligonucleotide  
15 by employing the same phosphoramidite chemistry used to  
polymerize the DNA bases. After purification of the  
linker-modified oligonucleotide, groups which selectively  
react with a primary alkyl amine can be added. These  
groups include the isothiocyanate derivative of eosin  
20 (EITC) or 9 amino acridine (AIT), or any number of other  
small molecules. Essentially identical chemistry is  
available for affixing a thiol group to the 5' terminus.

Fig. 6 shows dose dependent inhibition of HIV-1  
25 mRNA by Oligonucleotide mediated DNA triplexes.  
U937/HIV-1 cells (ATCC CRL 1593, American Type Culture  
Collection, Rockville, MD), infected with the HTLV-IIIB  
prototype strain of HIV-1 and cultured under conditions  
where >90% of the cells remained viable and contained  
30 HIV-1 mRNA as shown by *in situ* hybridization with the  
<sup>35</sup>S-labeled probe for the LTR of HIV-1, (NEP 200,  
DuPont, Wilmington, DE)] were incubated with each  
oligonucleotide at 0, 2, 6, 10, and 20 uM concentrations.  
Oligonucleotide was added to the culture supernatants at

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1       the initiation of incubation and again after 2 hours.  
Cells were harvested after 4 hours incubation, and washed  
with PBS before harvest of total cellular RNA using RNAsol  
(Cinna/Biotecx Laboratories International, Inc.,  
5       Friendswood, TX). Serial 2-fold dilutions were made from  
each RNA preparation (starting at 2.5 ug RNA) and equal  
amounts were applied to duplicate nylon membranes using a  
slot blot apparatus (Biorad). One blot was probed with  
10      the radiolabeled EcoR1-Hhal env fragment from the HIV-1  
containing plasmid pARV-7/2, while the other was probed  
with radiolabeled cDNA for  $\beta$ -actin. The resulting  
autoradiographs were then analyzed by densitometry. The  
density units expressed on the ordinate express the ratio  
of (env-probe density) / (actin-probe density).  $\Delta$   
15      represent HIV29par,  $\square$  represent HIV31 anti, and  $\square$   
represent random HIV29 isomer.

Fig. 7 shows the persistence of the effect of  
oligonucleotides on HIV infected H9 T cells. HIV-1  
20      infected U937 cells were cultured for 12 to 72 hrs. after  
the last addition of HIV31anti. The oligonucleotide was  
added at the initiation of the culture and at 2 hrs.  
thereafter to maintain a final concentration of 10 $\mu$ M.  
Cells were harvested at the indicated time points  
25      thereafter. Total cellular RNA was harvested and applied  
to duplicate nylon membranes in serial dilution with a  
slot blot apparatus. One replicate was probed with the  
HIV-1 env cDNA and the other with the cDNA for  $\beta$ -actin.  
The density units (ordinate) are expressed as the ratio of  
30      env to  $\beta$ -actin densitometry readings.  $\square$  represent  
HIV31 anti and  $O$  represent controls.

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1 Fig. 8 shows inhibition of viral mRNA by HIV29par  
in infected H9 cells. The densitometric analysis shows a  
decrease in specific viral message. H9 cells, infected  
with HTLV IIIB, were treated with oligomer (5 $\mu$ M) every  
5 two hours. At four and twelve hours the cells were  
harvested, washed with PBS, and the total cellular RNA was  
extracted. The hatched bars represent oligomer treatment  
and unhatched bars represent controls.

10 The drawings are not necessarily to scale.  
Certain features of the invention may be exaggerated in  
scale or shown in schematic form in the interest of  
clarity and conciseness.

15 DETAILED DESCRIPTION

It is readily apparent to one skilled in the art  
that various substitutions and modifications may be made  
to the invention disclosed herein without departing from  
20 the scope and spirit of the invention.

25 The term "synthetic oligonucleotides as used  
herein is defined as a molecule comprised of two or more  
deoxyribonucleotides or ribonucleotides, preferably more  
than ten. Its exact size will depend on many factors,  
including its specificity and binding affinity.

When referring to bases herein the term includes  
both deoxyribonucleic acids and ribonucleic acids. The  
30 following abbreviations are used: "A" refers to adenine as  
well as its deoxyribose derivatives, "T" refers to thymine  
as well as its deoxyribose derivative, "G" refers to  
guanine as well as its deoxyribose derivative, "C" refers  
to cytosine as well as its deoxyribose derivative, "X"

1 refers to xanthine as well as its deoxyribose derivative  
and "I" refers to inosine.

5 The "major groove" refers to one of the grooves  
along the outer surface of the DNA helix which is formed  
because the sugar-phosphate backbone extends further from  
the axis than the bases do. The major groove is important  
for binding of regulator molecules to specific DNA  
sequences.

10 A set of procedures have been established to  
design DNA or RNA oligonucleotides which bind specifically  
to a DNA target by colinear triplex formation. One  
embodiment of the present invention is a method for making  
15 a synthetic oligonucleotide which binds to a target  
sequence in duplex DNA forming a colinear triplex by  
binding to the major groove, said method comprising the  
steps of: scanning genomic duplex DNA and identifying  
nucleotide target sequences of greater than  
20 nucleotides, said target sequences having either about  
at least 65% purine bases or about at least 65% pyrimidine  
bases; and synthesizing said synthetic oligonucleotide  
complementary to said identified target sequence, said  
synthetic oligonucleotide having a G when the  
25 complementary location in the DNA duplex has a GC base  
pair, having a T when the complementary location in the  
DNA duplex has an AT base pair. In specific embodiments  
the synthetic oligonucleotide is selected from the group  
consisting of an oligonucleotide oriented 3' to 5' and  
30 binding anti-parallel to the about at least 65% purine  
strand and an oligonucleotide oriented 5' to 3' and  
binding parallel to the about at least 65% purine strand.  
The resulting oligonucleotide can be synthesized in gram  
quantities by the standard methods of solid phase  
35 oligonucleotide synthesis.

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1. The site-specific oligonucleotide procedure is divided into three parts:

- I. Oligonucleotide base sequence design.
- II. Analysis of the duplex target
- 5 III. Secondary chemical modification of the oligonucleotide.

I. Oligonucleotide base sequence design.

10 After identifying a DNA target with an interesting biological function, an oligonucleotide length must be chosen. There is a one to one correspondence between oligonucleotide length and target length. For example, a 27 base long oligonucleotide is required to bind to a 27 base pair long duplex DNA target. Under optimal conditions, the stability of the oligonucleotide-duplex DNA interaction generally increases continuously with oligonucleotide length. In the preferred embodiment, a DNA oligonucleotide in the range of about 20 to 40 bases is used. Oligonucleotides in this range usually have useful dissociation constants for their specific DNA target. The dissociation constants are in the range of about  $10^{-9}$  to  $10^{-8}$  molar.  
20 Oligonucleotides shorter than 20 bases display weaker and less specific binding to the target sequence and are thus less useful.

30 Oligonucleotide binding to duplex DNA is stabilized by binding to the purines in the underlying duplex. Once a DNA target has been identified, the more purine rich strand of the target area is defined as the "orienting" strand of the binding site. An oligonucleotide ligand was designed to bind either parallel or anti-parallel to the orienting strand. The

1 stability of the binding is dependent on the size of the  
oligonucleotide and the location in the genome. Sometimes  
the parallel is more stable than the anti-parallel while  
at other times the reverse is true or they are equally  
stable. In the preferred embodiment the method of  
5 designing a detailed sequence of an oligonucleotide ligand  
involves placing a T in the oligonucleotide whenever an AT  
base pair occurs in the duplex target, and placing a G in  
the oligonucleotide whenever a GC base pair occurs in the  
10 duplex target.

Examples of the orientation of bond donors and acceptors based on this oligonucleotide structure is displayed in Figures 2 and 3.

15 Another embodiment of the present invention includes a synthetic oligonucleotide for forming a colinear triplex with a target sequence in a duplex DNA when said target sequence is either about at least 65% purine bases or about at least 65% pyrimidine bases,  
20 comprising, a nucleotide sequence of at least about 20 nucleotides; said nucleotide sequence including G and T, wherein G is used when the complementary location in the duplex DNA is a GC base pair and T is used when the complementary location in the duplex DNA is an AT base pair; and said sequence selected from the group consisting of an oligonucleotide oriented 3' to 5' and binding anti-parallel to the about at least 65% purine strand in the duplex DNA target sequence and an oligonucleotide oriented 5' to 3' and binding parallel to the about at least 65% purine strand in the duplex DNA target sequence. Although molecules which include one or more bases which do not comply with this relationship can be fabricated, the binding affinity and site specificity of  
25  
30  
35

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1 these altered oligonucleotides will be reduced.  
Consequently the biological potency of these molecules  
will be inferior to the oligonucleotides having the G/GC  
and T/AT relationships.

5

Below is a schematic which demonstrates a target sequence, and oligonucleotides ligands which have been designed by the above design procedure.

10 Target Sequence (35bp)

5'-GGGAATTGGGCAGGTAAATTCGGGATAGGCGGTAA-3'  
3'-CCCTTAACCCGCCATTAAAGCCCTATCCGCCATT-5'

15 Parallel Synthetic Oligonucleotide

5'-GGGTTTGGGGGGTTTTTGGGGTTGGGGTTT-3' (par)

Anti-Parallel Synthetic Oligonucleotide

20 3'-GGGTTTGGGGGGTTTTTGGGGTTGGGGTTT-5' (anti)

If the synthetic oligonucleotide is constructed with a standard phosphodiester linkage, its binding affinity for the target would be near  $10^{-7}$  M under physiological conditions of salt, divalent ion concentration and temperature. Since the dissociation constant for oligonucleotide binding to a random DNA sequence population is near  $10^{-3}$  M for a 35 base oligonucleotide, the synthetic oligonucleotide affinity for the target would be approximately  $10^4$  times greater than for random sequence DNA under the same conditions.

1      II. Analysis of the duplex target.

5      If these procedures are followed to make a synthetic oligonucleotide, any duplex DNA sequence of about at least 65% purines can form a stable triplex.  
10     Within a DNA region, although the A+T content is not a significant consideration, duplex DNA sequences which have only purines on the template strand form complexes which in general, are characterized by enhanced stability. If we define n as the number of bases within the template strand which are purine and define (1-n) as the number of pyrimidine bases in the template, then the approximate dissociation constant can be predicted from the following semi-empirical formula:

15      $K = \exp^{-[0.4n + (0.2(1-n)/RT)]}$

20     This formula assumes near-physiological conditions in vitro, that is 0.05 M TRIS/HCl, 5mM MgCl<sub>2</sub>, 3mM spermine pH 7.8, 37°C. These conditions constitute the operating standard used in the design process.

25     This relationship predicts that an oligonucleotide designed to bind a 35 base long target sequence containing only purine bases in its template strand will form a triplex in which the oligonucleotide binds with a standard dissociation constant of about  $1 \times 10^{-10}$  M. This dissociation constant will be altered, however, when pyrimidine is in the template strand. In  
30     the above schematic representation where the template contains pyrimidine, the dissociation constant is  $3 \times 10^{-7}$  M.

1        This relationship is consistent with the  
observation that the free energy of triplex formation  
appears to increase in proportion to the span of the  
target-oligonucleotide interaction and the observation  
5        that the binding energy of a G to a GC base pair or a T to  
an AT base pair is dependant on base pair orientation  
relative to the template strand.

10      The molecular origin of that effect can be seen  
in Figure 2. It is evident that when the orienting strand  
comprises a series of purines, the bases in the  
complementary third strand form a contiguous stacked  
array. On the other hand, placing a pyrimidine in the  
orienting strand inverts the base pair. Thus, although  
15      third strand hydrogen bonding can still occur with  
parallel strand orientation upon forming a "Reverse  
Hoogsteen" bond at the site of inversion, it is associated  
with a dislocation of the path traversed by the third  
strand in the major groove. Thus for either an AT or GC  
20      base pair, approximately 0.4 kcal of favorable binding  
free energy results from third strand association at a  
purine site in the template, but only approximately 0.2  
kcal when the third strand binds to a site at which a  
purine to pyrimidine inversion has occurred.

25      III. Secondary chemical modification of the  
oligonucleotide.

30      A. One skilled in the art will recognize that a  
variety of synthetic procedures are available. In the  
preferred embodiment the oligonucleotides are synthesized  
by the phosphoramidite method, thereby yielding standard  
deoxyribonucleic acid oligomers.

1 Molecular modeling suggests that substitution of  
the non-hydrolyzable phosphodiester backbone in the  
oligonucleotide at elected sites may enhance the stability  
of the resulting triplex in certain instances. The  
5 phosphodiester analogues are more resistant to attack by  
cellular nucleases. Examples of non-hydrolyzable  
phosphodiester backbones are phosphorothioate,  
phosphoroselenoate, methyl phosphate, phosphotriester and  
the alpha enantiomer of naturally occurring  
10 phosphodiester. The thiophosphate and methyl phosphonate  
linkages are shown in Fig. 4. These non-hydrolyzable  
derivatives of the proposed oligonucleotide sequences can  
be produced, with little alteration of DNA target  
specificity.

15 Backbone modification provides a practical tool  
to "fine tune" the stability of oligonucleotide ligands  
inside a living cell. For example, oligonucleotides  
containing the natural phosphodiester linkage are degraded  
20 over the course of 1-2 hours in eukaryotic cells, while  
the non-hydrolyzable derivatives appear to be stable  
indefinitely.

B. Oligonucleotide hybrids provide another  
25 method to alter the characteristics of the synthetic  
oligonucleotides. Linkers can be attached to the 5'  
and/or 3' termini of the synthetic oligonucleotide. The  
linkers which are attached to the 5' terminus are usually  
selected from the group consisting of a base analogue with  
30 a primary amine affixed to the base plane through an alkyl  
linkage, a base analogue with a sulfhydryl affixed to the  
base plane through an alkyl linkage, a long chain amine  
coupled directly to the 5' hydroxyl group of the  
oligonucleotide and a long chain thiol coupled directly to

-20-

1       the 5' hydroxyl group of the oligonucleotide. The linker  
on the 3' terminus is usually a base analogue with a  
primary amine affixed to the base plane through an alkyl  
linkage or a base analogue with a sulfhydryl affixed to  
5       the base plane through a alkyl linkage. Affixation of a  
primary amine linkage to the terminus does not alter  
oligonucleotide binding to the duplex DNA target.

10     Once a linkage has been attached to the synthetic  
oligonucleotide a variety of modifying groups can be  
attached to the synthetic oligonucleotide. The molecules  
which can attach include intercalators, groove-binding  
molecules, cationic amines or cationic polypeptides. The  
modifying group can be selected for its ability to damage  
15     DNA. For example, the modifying group could include  
catalytic oxidants such as the iron-EDTA chelate, nitrogen  
mustards, alkylators, photochemical crosslinkers such as  
psoralin, photochemical sensitizers of singlet oxygen such  
as eosin, methylene blue, acridine orange and 9 amino  
20     acridine and reagents of direct photochemical damage such  
as ethidium and various pyrene derivatives.

25     For example an "aminolink", as supplied by  
Milligen (see Figure 5) works nicely. However, terminal  
coupling of any sort is likely to be equivalent. Once  
synthesized with an aminolink, the modified  
oligonucleotides can be coupled to any reagent which is  
specific for a primary amine, for example a succimidate or  
isothiocyanate moiety (Fig. 5).

30     In one embodiment, an "aminolink" coupling is  
used to affix the intercalating dyestuff 9 acridine  
isothiocanate to triplex forming oligonucleotides. The  
duplex binding affinity of the oligonucleotide-dye hybrid

1       is approximately 100-fold greater than the oligonucleotide  
binding affinity. Other embodiments include affixing  
eosin isothiocyanate to oligonucleotides. Since eosin  
isothiocyanate cleaves the DNA helix upon irradiation this  
5       hybrid oligonucleotide cuts the helix at its binding site  
when irradiated. This hybrid-oligonucleotide is useful  
for identifying the oligonucleotide binding site both in  
vitro and in vivo and potentially can be used as a  
therapeutic tool for selective gene target destruction.

10                  Photochemical reactivity is also achieved by  
affixation of psoralin derivatives to oligonucleotides  
through a 5' linkage. Psoralin binds covalently to DNA  
after irradiation, and as a consequence is a potent  
15                  cytotoxic agent. Thus, photochemical reactivity, with  
oligonucleotide sensitivity provides a tool to direct the  
toxic psoralin lesion to the oligonucleotide target site.

20                  Similar oligonucleotide coupling is used to  
target toxic chemical reactivity to specific DNA  
sequences. Examples include catalytic oxidants such as  
transition metal chelates and nucleases.

25                  Photochemical reactivity and/or toxic chemical  
agents can be used to permanently inhibit gene expression.

30                  In addition to chemical reactivity, modifications  
of oligonucleotides alter the rate of cellular uptake of  
the hybrid oligonucleotide molecules. The uptake process  
is rapid, but poorly understood. Terminal modification  
provides a useful procedure to modify cell type  
specificity, pharmacokinetics, nuclear permeability, and  
absolute cell uptake rate for oligonucleotide ligands.

1           C. Modified base analogues provide another means  
of altering the characteristics of the synthetic  
oligonucleotide. Although a purine rather than a  
pyrimidine, X is identical to T with respect to its  
5           capacity to form hydrogen bonds. Molecular modeling has  
shown that substitution of X for T in the above  
oligonucleotide design procedures, results in a modified  
triplex that is much more stable. The increased stability  
is due principally to enhanced stacking and to an  
10          enhancement of phosphodiester backbone symmetry within the  
ligand. Examples of base substitutions for T are X, I and  
halogenated X and I. G can be replaced by halogenated G.  
Furthermore, the 2' furanose position on the base can have  
a non-charged bulky group substitution. Examples of  
15          non-charged bulky groups include branched alkyls, sugars  
and branched sugars. In the preferred embodiment at least  
one base is substituted.

20          Molecular modeling suggests that oligonucleotide  
design will produce ligands with target affinity and  
specificity which exceeds that of even the most specific  
antigen-monoclonal antibody interaction.

25          Synthetic oligonucleotides have been designed to  
the transcription control region of the human c-myc  
protooncogene, to the regulation sequence of collagen  
I $\alpha$ , to bind to the TATA box segment of the chicken alpha  
actin gene, and to bind to an enhancer sequence within the  
early gene region of human HIV-I.

30          A further embodiment of the present invention is  
a method of inhibiting the growth of cells, comprising the  
step of administering a synthetic oligonucleotide in  
sufficient amount for cellular uptake and binding to the

1 target sequence, wherein said target sequence is  
positioned within the DNA domain adjacent to the RNA  
transcription origin. The synthetic oligonucleotide is as  
described above in the description of the design process.  
5 Uptake into the cells is rapid for these synthetic  
oligonucleotides and can be altered with the appropriate  
substitutions and modifications. Similarly the binding  
can be altered by appropriate changes to the synthetic  
oligonucleotide. The inhibition of cell growth can be  
10 used in the treatment of cancerous cells. Additions of  
the specific oligonucleotide will selectively inhibit cell  
growth. For example synthetic oligonucleotides to the  
c-myc gene can be used to inhibit some cancerous cell  
growth. Examples of synthetic oligonucleotide which  
15 inhibit c-myc expression include: 3'-TGGTGTGTGGGTTTGTTGGG  
GGGTGGGGGGTTTTTGGGTGGG-5' and/or  
3'-TGTGGTGGGTGGTTGGGTGGGTGGGTGGGTGGG-5' and/or  
5'-TTTGGTGTGGGGTGGGGTGGGTGGGTGGGTGGG-3' and/or  
3'-GGTTGGGTGGGTGGGTGGGTGGGTGGGTGGG-5' and/or  
20 5'-GGTTGGGTGGGTGGGTGGGTGGGTGGGTGGG-3' and fragments and  
analogues thereof.

Another embodiment includes a method of  
inhibiting the growth of pathogens comprising the step of  
25 administering a synthetic oligonucleotide in sufficient  
amount for cellular uptake and binding to the target  
sequence, wherein said sequence binds within the nucleic  
acid domain adjacent the RNA transcription origin. For  
example HIV-1 virus can be inhibited with a synthetic  
30 oligonucleotide which selectively binds to the viral LTR  
region. Specific examples of this synthetic  
oligonucleotide can include  
3'-GTTTTTGGGTGTTGGGTGTGGTGGTT-5' and/or  
35 5'-TGGGTGGGTGGGTGGGGGGGTGGGGGTGTGGGTGGGTG-3' and fragments  
and analogues thereof.

1 An additional embodiment includes a method of  
manipulating the structural protein content of epidermal  
tissue comprising the step of administering a synthetic  
oligonucleotide in sufficient amount for cellular uptake  
and binding to the target sequence. This includes  
5 inhibiting the various enzymes and regulating proteins in  
skin. For example, the collagen I $\alpha$  gene synthesis rate  
can be altered by using  
10 3'-TGGGTTGGGTGGTGGTGGGGGTGTGGTTGGTTGTGGGTTTT-5' and/or  
3'-GTGGGTTGGGTGGTGGTGGGGGTGTGGTTGG-5' and fragments and  
analogues thereof as the synthetic oligonucleotide.  
Similarly the collagenase gene can be inhibited by using  
15 5'GGTTGGGGTTGGTGTGTTTTTGTTGTGTGGTG-3' and/or  
5'-TTGTGGTTGTTTTGGTTGTGTGT-3, and fragments and  
analogues thereof.

20 The following examples are offered by way of  
illustration and are not intended to limit the invention  
in any manner. The synthetic oligonucleotides described  
in the examples can include any of the substitutions  
discussed earlier. The backbone, base, linkers and  
modifying groups can be added. These substitutions will  
enhance the affinity, the chemical stability, and the  
cellular uptake properties of the specific oligonucleotide  
25 treatments.

Example 1.

30 A. A Method For Arresting the Growth of Cancerous  
Tissue in Man, by Means of Intervention into the Program  
of c-myc Gene Expression.

35 Available evidence suggests that a family of  
tumors, including Burkitt's lymphoma and others, share a

-25-

1 common genetic lesion, which is evident as constitutive  
overproduction of the c-myc mRNA and its corresponding  
c-myc protein. Because the c-myc protein has been shown  
to be a critical element in the control of cell growth, it  
5 is believed that there may be a direct causal relation  
between the overproduction of c-myc protein and  
uncontrolled cancerous growth for such cells.

10 In both cancerous and normal cells, the c-myc  
gene possesses several target sequences within its 5'  
flanking sequence which satisfy the synthetic  
oligonucleotide design criteria. In a program of drug  
development, these target sequences and others are used as  
15 templates to direct oligonucleotide design. The purpose  
of these oligonucleotides is to selectively inhibit c-myc  
transcription, thereby repressing the uncontrolled growth  
of tumors with the c-myc lesion.

20 Three representative target sequences in the  
transcription control region of the human c-myc gene are  
shown below:

A. TARGET: THE TATA BOX FOR THE C-MYC GENE

25 DNA TARGET DUPLEX

-61

-16

5' -TCCTCTCTCGCTAATCTCCGCCACCGGCCTTTATAATGCGAGGG-3'

3' -AGGAGAGAGCGATTAGAGGCGGGTGGCCGGAAATATTACGCTCCC-5'

30 PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3' -TGGTGTGTGGGTTTGTTGCCCCGGTGGGGGGTTTTGGGTGGG-5'

-26-

## 1 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5' - TGGTGTGTGGTTTTGTGGGGGTGGGGGGTTTTTTGGGTGGG - 3'

5 B. TARGET: TRANSCRIPTION ACTIVATOR BINDING SITE -  
THE PRINCIPAL ACTIVATING PROTEIN BINDING SITE OF  
THE C-MYC GENE PROMOTER

Inappropriately high levels of c-myc gene expression are strongly associated with the incidence of a variety of human tumors. The triplex oligonucleotides described here were designed to selectively repress the expression of the c-myc gene in such tumors, thereby slowing tumor growth.

15 (1) DNA TARGET DUPLEX

-153

5' -TCTCCTCCCCACCTTCCCCACCCCTCCCCACCCCTCCCCA-3'  
20 3' -AGAGGAGGGGTGGAAAGGGGTGGGAGGGGTGGGAGGGGT-5'

## ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE

3' -GTGGTGGGTGGTTGGGTGGTGGGTGGGTGGGTGGGT-5'

-27-

## 1 (3) DNA TARGET DUPLEX (27bp)

-142 -115

5'-CCTTCCCCACCCCTCCCCACCCCTCCCCA-3'

3'-GGAAGGGTGGGAGGGGTGGGAGGGT-5'

5

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-GGTTGGGGTGGGTGGGTGGGTGGGT-5' (par)

## 10 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE

5'-GGTTGGGGTGGGTGGGTGGGTGGGT-3' (anti)

The 27 bp target duplex has 74% GC base pairs and 89% purine on the orienting strand. The Kdiss is ( $6 \times 10^{-10}$  M) for anti-parallel binding.

C. TARGET: SEQUENCE BETWEEN TATA BOX AND ACTIVATOR SITE IN A HIGHLY CONSERVED SEQUENCE AMONG THE VERTEBRATE c-myc GENE FAMILY.

## DNA TARGET DUPLEX

-87 -58

5'-AAAGCAGAGGGCGTGGGGAAAAGAAAAAGA-3'

25 3'-TTTCGTCTCCGCACCCCTTTCTTTTCT-5'

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-TTTGGTGTGGGGTGGGGTTTGTTTTGT-3'

30

## ANTIPARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-TTTGGTGTGGGGTGGGGTTTGTTTTGT-5'

1      The likely function of these sites, the position relative  
to the RNA transcription origin, and the oligonucleotide  
sequence which can be used as a c-myc specific treatment  
are shown. One skilled in the art will readily recognize  
5      that as the molecular genetics of the c-myc gene is  
elucidated in greater detail, the list of target sequences  
within the 5' flanking region will be expanded, by  
application of the above design criteria.

10     Both synthetic oligonucleotides A and B  
specifically interact within the target duplex to inhibit  
tumor growth, by means of specific repression of c-myc  
transcription. The specific method of inhibition of  
oligonucleotide C is unknown.

15     One skilled in the art will readily recognize  
that oligonucleotides for other genes involved in human  
tumors can be similarly designed. The procedure is only  
limited by the available molecular sequence data.

20                   Example 2.

25     A Method for Manipulating the Structural Protein  
Content of Epidermal Tissues, for the Purpose of Altering  
Tissue Appearance and Wound Healing.

30     The structural proteins which define the mechanical  
properties of skin are well known. The molecular  
structure of the collagen and elastin proteins and their  
corresponding proteases, collagenase and elastase, have  
been intensley studied. These proteins are under the  
control of an elaborate program of regulation, which  
appears to change during the wound healing process and as  
a result of the aging process. The molecular structure is

-29-

1 sufficiently defined to consider treatments based upon  
gene-specific intervention into the pattern of structural  
protein synthesis and/or enzymatic degradation.

5 Data suggest that the change in the mechanical  
properties of skin which accompanies aging (wrinkling,  
etc.) is due in part to an age-specific change in the  
relative abundance of the collagens and other structural  
proteins. Interference with the synthesis and/or  
10 selective degradation of these proteins by drug treatment  
can reestablish a distribution which approximates that of  
younger tissue, and thus the effects of aging can be  
partially reversed.

15 A program of synthetic oligonucleotide design,  
based upon manipulation of collagen I synthesis in human  
skin is described below. By altering the relative protein  
concentrations the structure and mechanical properties of  
skin can be altered. Thus the synthetic oligonucleotide  
20 can be used as a therapeutic agent to alter the skin aging  
process or to alter the wound healing process. One  
skilled in the art will readily recognize that the  
concepts can be extended to other collagens, to other skin  
proteins and to their complementary proteases based upon  
25 the availability of the necessary genetic data.

Representative target sequences in the  
transcription control region of the human alpha 1(I)  
collagen gene, the likely function of those sites, their  
30 position relative to the RNA transcription origin, and the  
synthetic oligonucleotide sequence designed for collagen  
specific treatment as shown below. As the molecular  
genetics of the collagen gene develops, the list of target  
sequences within the 5' flanking region will be expanded.

-30-

## 1 A. TARGET: THE CAT BOX FOR THE COLLAGEN GENE

## DNA TARGET DUPLEX

5 -168

-124

5'-TCCCTTCCTCCTCCTCCCCCTCTCCATTCCAACTCCAAATT-3'3'—AGGAAAGGGAGGAGGAGGGGGAGAGGTAAGGTTGAGGGTTAA-5'

## SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

10

3'—TGGGTTGGGTGGTGGTGGGGGTGTGGTTTGAGGGTTTT-5'

## B. TARGET: ENHANCER FOR THE COLLAGEN GENE

15

## DNA TARGET DUPLEX

-294

-264

5'-CCCTACCCACTGGTTAGCCCACGCCATTCT-3'20 3'—GGGATGGGTGACCAATCGGGTGCAGTAAGA-5'

## SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

25 3'—GGGTTGGGTGTGGTTGGGGTGGGGTTGG-5'

25

C. TARGET: HIGHLY CONSERVED POLYPURINE SEGMENT  
WHICH OCCURS NEAR -200 IN ALL COLLAGENS

## 30 DNA TARGET DUPLEX

-177

-136

5'-CTCCCTTCCTCCTCCTCCCCCTCTCCATTCC-3'

3'—GAGGGAAGGGAGGAGGAGGGGGAGAGGTAAGG-5'

35

## 1      SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3' -GTGGGTTGGGTGGTGGTGGGGGTGTGGTTTGG-5'

5

Synthetic oligonucleotides A and B inhibit type I collagen protein synthesis. The process includes the specific repression of collagen RNA transcription. The method of inhibition of the C synthetic oligonucleotide is not known. The effect on protein synthesis of skin proteins can be seen by adding sufficient amounts of the synthetic oligonucleotide for uptake into cultured human fibroblasts.

15

Next, two representative target sequences are described in the transcription control region of the human collagenase gene, the function of these sites, their position relative to the RNA transcription origin, and the oligonucleotide sequence designed as a collagen specific treatment. As the molecular genetics of the collagenase gene develops, the list of target sequences within the 5' flanking region will be expanded.

25

## D.      TARGET: THE TATA BOX FOR THE COLLAGENASE GENE

## DNA TARGET DUPLEX

-48

-16

5' -GGAAGGGCAAGGACTTATATATACAGAGGGAG-3'

30

3' -CCTTCCCGTCCTGAGATATATGTCTCCCTC-5'

## SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5' -GGTTGGGGTTGGTGTGTTTTTGTTGTGGGTG-3'

1 E. TARGET: THE INDUCIBLE ENHANCER FOR THE  
COLLAGENASE GENE. CONFIRMS TPA TUMOR PROMOTOR  
RESPONSIVENESS

## 5 DNA TARGET DUPLEX

-91 -64  
-AAGAGGATGTTATAAAGCATGAGTCAGA-3  
-TTCTCCTACAATTTCGTACTCAGTCT-5

10 SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5' -TTGTGGTTGTTTTTTGGTTGTGTGT-3'

15           The D synthetic oligonucleotide inhibits  
collagenase protein synthesis. The process includes  
specific repression of collagenase RNA transcription. The  
E synthetic oligonucleotide causes loss of TPA  
sensitivity, and a subsequent repression of collagenase  
syntheses in the presence of promotores such as TPA. This  
process includes specific repression of collagenase RNA  
transcription. Synthetic oligonucleotide interaction will  
cause collagen protein levels in the cell to rise, as  
collagenase levels fall. The clinical effect of the  
increase should cause a useful alteration of the  
mechanical properties of skin. The effects can be seen by  
adding sufficient amounts of oligonucleotide for cellular  
uptake to cultured human fibroblasts.

30 One skilled in the art will readily appreciate  
that these concepts can be extended to other genes which  
are known to be involved in skin development, repair and  
aging and is only limited by the available molecular  
genetic data.

35

## Example 3

A Method to Repress the Growth of Human HIV-1 Virus,  
by means of Oligonucleotide Binding to Target Sites  
within the HIV-1 LTR.

The HIV-I virus is known to be the causative agent in human acquired immune deficiency syndrome (AIDS). The long terminal repeat of the HIV-1 virus is known to possess several DNA segments within the LTR region which are required for transcription initiation in a human T-cell host. The synthetic oligonucleotides selectively repress HIV-1 mRNA synthesis in a human host cell, by means of triplex formation upon target sequences within the viral LTR. Repression of RNA synthesis results in the reduction of the growth rate of the virus. This could result in the slowing of the infection process or the repression of the transition from latency to virulent growth. Most of the sites within the LTR will comprise target sites for drug (oligonucleotide) intervention. There is no wasted DNA in the small, highly conserved LTR region.

Representative target sequences in the transcription control region of the human HIV-1 LTR, the likely function of these sites, their position relative to the RNA transcription origin, and the oligonucleotide sequence designed as a HIV-I specific treatment are shown below. As the molecular genetics of HIV-I develops, the list of target sequences within the LTR and elsewhere will be expanded.

1           In all instances, both the parallel and  
antiparallel isomers are described. The reason is that,  
although one or the other will always display the better  
binding affinity in vitro, the efficacy of each must be  
5           tested in vivo to make the final decision.

A. TARGET: THE 5' END OF THE HIV-1 LTR DOMAIN

DNA Target Duplex (25bp, 92% Purine)

10           -470    -446  
5'-AAAAAGAAAAGGGGGGACTGGAAGGG-3'  
3'-TTTCTTTCCCCCTGACCTCCC-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

15           5'-TTTGTTTGGGGGTGTGGTGGG-5' (HIV1par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

20           3'-TTTGTTTGGGGGTGTGGTGGG-5' (HIV1anti)

B. TARGET SITE: A segment of the negative HIV1  
regulatory domain, with similarity to a homologous domain  
in interleukin 2 gene.

25           DNA Target Duplex (33bp, 88% purine)

-293    -261  
5'-AGAGAAGGTAGAACAGAGGCCAATGAAGGAGAGAA-3'  
3'-TCTCTCCATCTCTCCGGTTACTCCTCTCTT-5'

30           PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-TGTGTTGGTTGTTGTGGGTTTGTTGGTGTGTT-3' (HIV2par)

-35-

## 1 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-TGTGTTGGTTGTTGTGGGGTTTGTTGGTGTGTT-5' (HIV2anti)

5 C: TARGET SITE: A site near the center of the LTR.

DNA Target Duplex (25bp, 88% purine)

10 -229 -205

9327 9351

5'-GGGATGGAGGACGCCGGAGAAAGAAG-3'

3'-CCCTACCTCCTGCCTCTTCTTC-5'

## 15 PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

15 5'-GGGTTGGTGGTGGGGGTGTTGTTG-3' (HIV3par)

## 20 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

20 3'-GGGTTGGTGGTGGGGGTGTTGTTG-5' (HIV3anti)

## D. TARGET SITE:

25 Binding site for the Spl-line transcription activator.

(1) DNA Target Duplex (36bp, 78% purine)

30 -80 -51

30 5'-AGGGAGGGCGTGGCCTGGCGGGACTGGGGAGTGGCG-3'

3'-TCCCTCCGCACCGGACCCGCCCTGACCCCTCACCGC-5'

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

-36-

5'-TGGGTGGGGTGGGGTGGGGGGGTGTGGGGTGTGGGG-3' (HIV4par) or  
(HIV36par)

## ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5

3'-TGGGTGGGTTGGGTGGGGGGTGTGCCCTCTGGGG-5' (HIV4anti) or  
(HIV36anti)

The HIV4 par also functions if TG is added to the 3' end  
to make HIV38 par.

E. TARGET: BINDING SITE FOR THE TRANSCRIPTION ACTIVATOR REGION (tar); THE DOWNSTREAM HALF OF THE tar SITE

#### **DNA TARGET DUPLEX (29-31bp, 72% purine)**

-16 - 13

5'-CTTTTTGCCTGTACTGGCTCTCTCTGCTT-3'

3'-GAAAAAACGGACATGACCCAGAGAGGAGTTC-5'

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-GTTTTTGGGTGTTGTGGGTGTCTCTGCTT-5' (antisense)

## ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE

25

5'-GTTTTGGGTGTTGTGGGTGTGTGCTTC-3' (reverse strand)

The oligonucleotides, HIV29par and HIV31anti, were designed as previously described herein. HIV31anti also functions if bases two TG are removed from the 3' end. The relative mobility and DNA footprint analyses of both oligonucleotides show binding with high affinity to target proviral sequences, *in vitro*.

1           HIV-1 infected U937 cells, a monocyteoid line,  
were treated with up to 20 $\mu$ M with either HIV29par,  
HIV31anti, or a random isomer of HIV29 with no detectable  
5           *in vitro* affinity for the target sequence. Significant  
inhibition of viral mRNA production, as shown by the  
decrease in the relative concentrations of env as compared  
to  $\beta$ -actin mRNA, was achieved at a dose of 10 $\mu$ M of  
either oligonucleotide ( $p < .01$ , paired t-test, figure  
6). No additional suppression was observed at 20 $\mu$ M.  
10          The random isomer of HIV29 did not inhibit viral mRNA  
synthesis, even at 20 $\mu$ M, confirming the specificity of  
the suppression achieved with HIV29.

15          We found that when U937/HIV-1 cells were  
incubated in media containing 0.6 uM  $^{32}$ P-labeled  
HIV29par, the cells were able to rapidly sequester the  
oligomer in concentrations exceeding that of the media.  
Assuming an average cell volume of 350 fL, it was  
determined that the intracellular concentration increased  
20          from 2.4 $\mu$ M after 10 minutes to a plateau of about 6 $\mu$ M  
after 2 hours. The oligonucleotides had a prolonged  
effect on HIV-1 transcription in that two treatments,  
spaced two hours apart, inhibited viral mRNA synthesis for  
up to 72 hrs (figure 7). Further studies showed the  
25          effect of tar sequence specific oligonucleotides on  
infected T cells. HIV29par was used to treat HIV-infected  
H9 T cells. Treatment every 2 hrs. with 5 $\mu$ M effectively  
suppressed mRNA synthesis in HIV-1 infected H9 T cells at  
2 and 12 hours.

30          Thus, the evidence shows that the  
oligonucleotides designed to bind within the major groove  
of the DNA helix, and form triplexes with specific gene  
sequences in the tar region of the HIV-1 provirus are

-38-

1 readily taken up by HIV-1 infected cells and selectively  
2 suppress synthesis of HIV-1 mRNA without concomitant  
3 suppression of mRNA for  $\beta$ -actin, which constitutive  
4 expressed in these cells. With inhibition of viral mRNA  
5 synthesis, translation of virus-encoded proteins is also  
6 suppressed. Inhibition of viral mRNA depended on the dose  
7 of oligonucleotide added; maximum inhibition occurred at  
8 concentrations  $\geq 10\mu M$ . The oligonucleotides designed  
9 to bind to specific sequences in the DNA duplex and form  
10 colinear triplex with the targeted sequences provide an  
11 efficient and highly specific agent for regulating gene  
12 expression, such agents provide a new class of rationally  
13 designed chemotherapeutic agents for controlling virus  
14 replication and other processes depend upon new mRNA  
15 production.

20 The synthetic oligonucleotides in A through E  
will inhibit HIV-I mRNA synthesis, hence viral growth.  
The process includes specific repression of RNA  
transcription from the viral LTR.

25 One skilled in the art will readily recognize  
that these concepts can be extended to other genes which  
are known to be involved in the infection process by which  
HIV-I and other viruses act.

#### Example 4

30 A Method for Altering Chicken Skeletal Actin  
Transcription.

35 A representative target sequence in the  
transcriptions control region of the chicken skeletal  
alpha actin gene, the function of that site, its position

-39-

1 relative to the RNA transcription origin, and the  
oligonucleotide sequence which would be designed as an  
actin specific treatment are shown below. As the  
molecular genetics of the actin gene develops, the list of  
5 target sequences within the actin control region will be  
expanded.

A. TARGET: THE TATA BOX FOR THE CHICKEN SKELETAL  
ALPHA ACTIN GENE

10 DNA TARGET DUPLEX

-30 -4  
5' - GATAAAAGGCTCCGGGCCGGCGGG-3'  
3' - CTATTTCCGAGGCCCGGCCGCC-5'

15 SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5' - GTTTTTTGGGTGGGGGGGGGGGGGGGG-3'

20 This synthetic oligonucleotide molecule inhibits  
actin protein synthesis, by specific repression of RNA  
transcription. This inhibition can be assessed in  
cultured chicken myoblasts. The intact chicken will show  
a change in the quality of actin and other muscle proteins  
25 whose synthesis is strongly coupled to actin expression.  
The practical result of this change will be an alteration  
of the properties of chicken meat.

30 One skilled in the art will readily appreciate  
that these concepts can be extended to other genes which  
are known to be involved in muscle growth and development,  
and is limited by the available molecular genetic data.

-40-

## 1 Example 5

INTERLEUKIN 2 ALPHA CHAIN RECEPTOR

5 TARGET: TRANS PROMOTOR REGION

DNA Target Duplex (28bp)

-273 -246

10 5' -AACGGCAGGGGAATCTCCCTCTCCTTT-3'  
3' -TTGCCGTCCCCTTAGAGGGAGAGGAAAA-5'

Parallel Synthetic Oligonucleotide

15 5' -TTGGGGTGGGGTTTGTGGGTGTGGTTTT-3' (IL28par)

Anti-Parallel Synthetic Oligonucleotide

20 3' TTGGGGTGGGGTTTGTGGGTGTGGTTTT-5' (IL28anti)

The 28bp target is comprised of 54% G+C base pairs and is  
61% purine on the orienting strand. The Kdiss for the  
parallel stand is  $1.5 \times 10^{-7}$  and the Kdiss for the  
antiparallel is  $8 \times 10^{-7}$ .

25

## Example 6

A Sequence For Dispersing Plaque Formation  
in Alzheimers Disease

30

The APP770 Gene is the precursor protein  
responsible for production of plaque in Alzheimers disease.

35

-41-

## 1 A. TARGET SITE: DOWNSTREAM TATA BOX SITE

## DNA Duplex Target

5 -712 -679  
5' -AAAAACAAACAAAAATATAAGAAAGAAACAAAA-3'  
3' -TTTTGTTGTTTATATTCTTCCTTGTTT-5'

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

10 10 5' -TTTTGTTGTTTCTTCCTTCCTT-3' (APP1par)

## ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

15 15 3' -TTTTGTTGTTTCTTCCTTCCTT-5' (APP1anti)

## B. TARGET: UNKNOWN

## 20 DNA Duplex Target

-618 -590  
5' -TCCTGCCCTGCTCCTTGGTTGTT-3'  
3' -AGGACCGGAACGAGGAAACCAAGCAAGA-5'

25 25 PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3' -TGGTGGGGTTGGTGGTTGGTTGGTGT-5' (APP2par)

30 30 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5' -TGGTGGGGTTGGTGGTTGGTTGGTGT-3' (APP2anti)

-42-

## C. TARGET: UNKNOWN

## DNA Duplex Target

5            -477            -440  
5' -TTCTCATTCTCTTCCAGAAACGCCTGCCACCTCTCC-3'  
3' -AAGAGTAAGAGAAGGTCTTGCGGACGGGGTGGATAGG-5'

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

10           3' -TTGTGTTTGTGTTGGTGTGTTGGGGTGGGTGGTGG-5' (APP3par)

## ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

15           5' -TTGTGTTTGTGTTGGTGTGTTGGGGTGGGTGGTGG-3' (APP3anti)

## D. TARGET: UNKNOWN

## DNA Duplex Target

20           -434            -407  
5' -GAGAGAAAAAACGAAATGCGGATAAAA-3'  
3' -CTCTCTTTTGCTTACGCCTATTTT-5'

25           PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5' -GTGTGTTTTGGTTTGGGGTTTTT-3' (APP4par)

30           ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3' -GTGTGTTTTGGTTTGGGGTTTTT-5' (APP4anti)

## 1      E.            TARGET: UNKNOWN

## DNA Duplex Target

5                    -286                    -252  
5 ' -CTCACCTTCCCTGATCCTGCACCGTCCCTTCCT-3 '  
3 ' -GAGTGGAAAGGGACTAGGACGTGGCAGGGAGAGGA-5 '

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

10                  3 ' -GTGTGGTTTGGGTGTTGGTGGTGGGTGGGTGGT-5 ' (APP5par)

## ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

15                  5 ' -GTGTGGTTTGGGTGTTGGTGGTGGTGGGTGGTGGT-3 ' (APP5anti)

## F.            TARGET: UNKNOWN

## 20            DNA Duplex Target

-264                    -230  
5 ' -CCGTCCCTCTCCTGGCCCCAGACTCTCCCTCCC-3 '  
3 ' -GGCAGGGAGAGGACCAGGGTCTGAGAGGGAGGG-5 '

25            PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE  
3 ' -GGGTGGGTGTGGTGGGGGTGTGTGGGTGGG-5 ' (APP6par)

30            ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE  
5 ' -GGGTGGGTGTGGTGGGGGTGTGTGGGTGGG-3 ' (APP6anti)

-44-

## 1 G. TARGET: UNKNOWN

## DNA Duplex Target

5 -200 -177  
5'-GGGGAGCGGGAGGGGGCGCGTGGG-3'  
3'-CCCTCGCCTCCCCCGCGCACCC-5'

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

10 5'-GGGGTGGGGTGGGGGGGGTGGG-3' (APP7par)

## ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

15 3'-GGGGTGGGGTGGGGGGGGTGGG-5' (APP7anti)

## H. TARGET: UNKNOWN

## 20 DNA Duplex Target

-40 -9  
5'-CTGCCTGGCTCTGAGCCCCGCCGCCGCTC-3'  
3'-GAGCGGACCGAGACTCGGGCGGCGCGAG-5'

## 25 PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

30 3'-GTGGGGTGGGTGTGTGGGGGGGGGGGGTG-5' (APP8par)

## 30 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-GTGGGGTGGGTGTGTGGGGGGGGGGGGTG-3' (APP8anti)

1

## Example 7

## THE EGFR PROMOTOR DOMAIN

5           Inappropriately high expression of the epidermal  
growth factor gene (EGFR) has been implicated as crucial  
to the development of cancers and several skin diseases  
(psoriasis). The synthetic oligonucleotides described  
10          below were designed to selectively repress the expression  
of the EGFR gene in such diseases.

## A.           TARGET: SP1 BINDING SITE

## 15          DNA Duplex Target

              -109                           -83  
5' -TCCGCCAGTCCCCGCCTCGCCGCC-3'  
3' -AGGCCTCAGGGCGGAGCGCGG-5'

## 20          PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3' -TGGGGGGTGTGGGGGGTGGGGGG-5' (EGFR<sub>1par</sub>)

## 25          ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5' -TGGGGGGTGTGGGGGGTGGGGGG-3' (EGFR<sub>1anti</sub>)

30

35

## 1        B.           TARGET    SPI BINDING SITE

## DNA Duplex Target

5                          -307                          -281  
5 ' -TCCCTCCTCCTCCGCCCTGCCTCCC-3 '  
3 ' -AGGGAGGAGGAGGGCGGGACGGAGGG-5 '

## 10                        PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

10                        3 ' -TGGGTGGTGGTGGGGGGGTGGGTGGG-5 ' (EGFR2par)

## 15                        ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

15                        5 ' -TGGGTGGTGGTGGGGGGGTGGGTGGG-3 ' (EGFR2anti)

## 20        C.           TARGET:    SPI BINDING SITE

## 20        DNA Duplex Target

25                          -352                          -317  
5 ' -TTCTCCTCCTCCTCTGCTCCTCCGATCCCTCCTCC-3 '  
3 ' -AAGAGGAGGAGGAGACGAGGAGGGCTAGGGAGGAGG-5 '

## 25                        PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

30                        3 ' -TTGTGGTGGTGGTGTGGTGGTGGGGTTGGGTGGTGG-5 ' (EGFR3par)

## 30                        ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

35                        5 ' -TTGTGGTGGTGGTGTGGTGGTGGGGTTGGGTGGTGG-3 ' (EGFR3anti)

D. TARGET: NUCLEASE SENSITIVE DOMAIN REQUIRED FOR  
EGFR EXPRESSION

### DNA Duplex Target

5

5' -TTCTCCTCCCTCCTCGCATTCTCCTCCTCCTCT-3'

3'-AAGAGGAGGGAGGAGGAGCGTAAGAGGAGGAGGAGA-5'

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-TTGTGGTGGGTGGTGGTGGGTGGGTGGTGGTGGTGGTGT-5' (EGFR4par)

## ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

15

5' -TTGTGGTGGGTGGTGGTGGGTGGGTGGTGGTGGTGGTGGT-3' (EGFR4anti)

### Example 8

20

## THE GST<sub>pi</sub> GENE

## Overexpression of the enzyme

25 glutathione-s-transferase pi has been implicated as being  
responsible for the broad-range drug resistance which  
developes in a variety of cancers. The synthetic  
oligonucleotides described below are designed to repress  
GST-pi expression, thereby sensitizing cancerous tissue to  
traditional drug chemotherapy.

30

A. TARGET SITE: The target domain comprises the consensus binding sequences for the transcription activating factors AP1 and Sp1. Synthetic Oligonucleotides targeted against this will repress GSTpi transcription by means of competition with AP1 and Sp1.

## 1 DNA Duplex Target

-68 -39

5' -GACTCAGCACTGGGGCGGAGCGGGGCGGGA-3'

5 3' -CTGAGTCGTGACCCGCCTGCCCGCCCT-5'

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5' -GTGTGTGGTGTGGGGGGGTGGGGGGGGGT-3' (GST1par)

10 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3' -GTGTGTGGTGTGGGGGGGTGGGGGGGGGT-5' (GST1anti)

15 B. TARGET SITE: An enhancer-like polypurine sequence. A synthetic oligonucleotide targeted against this site will repress GSTpi transcription by means of competition with the enhancer.

20 -227 -204

5' -GGGGACCTGGAAAGAGGGAAAGG-3'

3' -CCCCTGGACCCTTTCTCCCTTCC-5'

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

25 5' -GGGTGGTGGTTGTGGTTGG-3' (GST2par)

## ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

30 3' -GGGTGGTGGTTGTGGTTGG-5' (GST2anti)

35 An unusual repetitive DNA segment. No function has been ascribed to this segment yet. However, it is within the control domain and may play a role in transcription initiation.

## 1 DNA Duplex Target

-499

-410

5'-AAAATAAAATAAAATAAAATAAAATAAAAT-3'

5 3'-TTTATTATTATTTATTATTTATTATTTA-5'

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-TTTTTTTTTTTTTTTTTTTTTTTTTT-3' (GST3par)

## 10 15 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-TTTTTTTTTTTTTTTTTTTTTTTT-5' (GST3anti)

## 15 Example 9

## The HMGCoA REDUCTASE GENE

20 HMGCoA Reductase is the enzyme which defines the rate limiting step in cholesterol biosynthesis. Its molecular genetics has been studied to understand the control of cholesterol synthesis. The described synthetic oligonucleotides will intervene in the program of cholesterol synthesis by means of modulating the transcription of HMGCoA.

25 A. TARGET SITE: The target is binding site for a repressor protein that appears to mediate end-product inhibition of transcription by cholesterol. The synthetic oligonucleotide is a synthetic repressor of HMGCoA expression, as an agonist of the cellular repressor.

-50-

## 1 DNA Duplex Target

-167

-135

5 ' -GGTGAGAGATGGTGC GG TGCCC GTT CTCC GCCC -3 '

5 3 ' -CCACTCTCTACCACGCCACGGGCAAGAGGC GGGG -5

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3 ' -GGTGTGTGTTGGTGGGGTGGGGGTTGTGGGGGG -5 ' (HMGCOA1par)

## 10 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5 ' -GGTGTGTGTTGGTGGGGTGGGGGTTGTGGGGGG -3 ' (HMGCOA1anti)

15 B. TARGET SITE: The target is a binding site for protein that appears to activate transcription of HMGCoA. The synthetic oligonucleotide against this site is a synthetic repressor of HMGCoA expression, as an antagonist of the cellular protein which binds to the target.

## 20 DNA Duplex Target

-134

-104

5 ' -GGGTGCGAGCAGTGGCGGGTTGTTAAGGCGA -3 '

25 3 ' -CCCACGCTCGTCACCCGCCAACAAATTCCGCT -5 '

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

30 5 ' -GGGTGGGTGGTGTGGGGGGTTGTTTGGGGT -3 ' (HMGCOA2par)

## 30 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

35 3 ' -GGGTGGGTGGTGTGGGGGGTTGTTTGGGGT -5 ' (HMGCOA2anti)

1 C. TARGET SITE: The target is a binding site for a  
protein that appears to activate transcription of HMGCoA  
by binding to the "TATA box" domain. A TFO against this  
site is designed to be a synthetic repressor of HMGCoA  
expression, as an antagonist of the cellular protein which  
5 binds to the TATA box target.

### DNA Duplex Target

10 -41 -6  
5' -AGGC GATCGGACGATCCTTCTTATTGGCGGCCCT-3'  
3' -TCCGCTAGCCTGCTAGGAAAGAATAACCGCCGGGA-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

15 3'-TGGGGTTGGGTGGTTGGTTGGTTGGGGGGGGT-5' (HMGCOA3 par)

## ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

20 5'-TGGGGTTGGGTGGTTGGTTGGTTGGGGGGGGT-3' (HMGCOA3 anti)

#### Example 10

## Nerve Growth Receptor (NGFR)

The NGFR gene encodes a cell surface receptor required for nerve cell proliferation. It is overexpressed in neuroblastoma and melanomas. Triplex oligonucleotides are designed to repress the growth of those cancerous tissues. Activation of the gene would be a precondition of activation of nerve cell regeneration. The mRNA start site is at -122 in this number scheme.

-52-

## 1 A. TARGET SITE: Consensus Sp1 binding site

## DNA Duplex Target

5 -323 -290  
5' -GGGAACTGGGTACCAGGGCGGGATGGGTGAGAGG-3'

3' -CCCTTGACCCATGGTCCCCCCTACCCACTCTCC-5'

## 10 PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5' -GGGTTGTGGGTTGGTGGGGGGTTGGGTGTGTGG-3'  
(NGFR1par)

## 15 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3' -GGGTTGTGGGTTGGTGGGGGGTTGGGTGTGTGG-5' NGFR1ap

## B. TARGET SITE: Consensus Sp1 binding site.

## 20 DNA Duplex Target

-309 -275  
5' -AGGGCGGGATGGGTGAGAGGGCTCTAAGGGACAAGG-3'

25 3' -TCCCGCCCTACCCACTCTCCGAGATTCCCTGTTCC-5'

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

30 5' -TGGGGGGGTTGGGTGTGGGTGTTGGGTGTTGG-3'  
(NGFR2par)

## ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

35 3' -TGGGGGGGTTGGGTGTGGGTGTTGGGTGTTGG-5'  
(NGFR2anti)

1 C. TARGET SITE: Domain flanking consensus Spl  
binding sites.

DNA Duplex Target

5 -285 -248  
5'-AAGGGACAAGGCAGGGAGAACGCGCACGGGTGCGGGAA-3'

3'-TTCCCTGTTCCGTCCCTCTCGCGTGCCACGCCCTT-5'

10 PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-TTGGGTGTTGGGTGGGTGTTGGGTGGGTGGGGTT-3'  
(NGFR3par)

15 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-TTGGGTGTTGGGTGGGTGTTGGGTGGGTGGGGTT-5'  
(NGFR3anti)

20 D. TARGET SITE: Domain flanking consensus Spl  
binding sites.

DNA Duplex Target

25 -243 -216  
5'-CCCTCCCTTGCCCTCTGCTTCCCACCCC-3'

3'-GGGAGGGAAACGGAGACGAAGGGTGGGG-5'

30 PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-GGGTGGTTGGGTGTGGTTGGGTGGGG-3' (NGFR4par)

35 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-GGGTGGGTTGGGTGTGGTTGGGTGGGG-5' (NGFR4anti)

**TARGET SITE:** Consensus Spl binding site.

### DNA Duplex Target

-187-

- 154

5' -GGGGGTGGGCGGGCTGGCGGGGCGGAGGGCGGGG-3'

10 3'-CCCCCACCCGCCGGACCGCCCCGCCCTCCGCCCCCC-5'

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5' -GGGGGTGGGGGGGGTGGGGGGGGGTGGGGGGGG-3'

(NGFR5par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-GGGGGTGGGGGGGTGGGGGGGGGTGGGGGGGG-5'

(NGFR5anti)

### Example 11

## HERPES SIMPLEX VIRUS 1: DNA Polymerase and DNA binding protein

25

HSV-1 is responsible for a variety of skin lesions and other infections. The triplex oligonucleotide are designed to bind directly to the promotor region of the genes which encode the viral DNA polymerase and DNA binding protein, thereby arresting viral replication. Both genes occur at 0.4 map units and flank the replication origin oriL. Numbering below is in terms of the polypeptide start site for each gene.

35

A. TARGET SITE This site is in the 5' flanking sequence of the DNA polymerase gene. The Angelotti strain has three base changes relative to strain 17.

5 (l) Strain 17

### DNA Duplex Target

-60 -26  
10 5'-TTTTCTCTCCCCCTCCCCACATTCCCCTTTT-3'

3' -AAAAAGAGAAGGGGGAGGGTGTAAAGGGAGAAA-5'

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

15           3' -TTTTTGTGTTGGGGGTGGGTGTGGGGGTGTT-5'  
             (HSVPOL17par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

20           5' - TTTTGTTGGGGGGTGGGTGTGGGGGTGTT-3'  
(HSVpol17anti)

### (3) Strain Angelotti

25 -62 -26  
5'-TTTTTCTCTCCCCCCCCCTCCCCACATCCCCCCTCTT-3'

3' -AAAAAGAGAAGGGGGGGAGGGGTGTAGGGGGAGAAA-5'

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-TTTTTGTGTTGGGGGGTGGGTGTTGGGGGTGTT-5'  
(HSV<sub>1</sub>POL1par)

35 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5 A. TARGET SITE: This site is in the 5' flanking sequence of the DNA binding protein gene for strain 17.

-82 -118

5' -AAAATCCGGGGGGGGCGGCGACGGTCAAGGGGAGGG-3'

10 3'-TTTTAGGCCCGCCGGCTGCCAGTTCCCCCTCCC-5'

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

15 5'-TTTTGGGGGGGGGGGGGGGGGGTGGGTGGGTGGG-3'  
(HSV<sub>pol</sub>2par)

## **ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE**

20 3'-TTTTTGGGGGGGGGGGGGGGGGGTGGGTGTTGGGTGGG-5'  
(HSV<sub>1</sub>POL2anti)

### Example 12

## HERPES SIMPLEX VIRUS 1: origin of replication

HSV-1 is responsible for a variety of skin lesions and other infections. The triplex oligonucleotides are designed to bind directly to the two classes of HSV-1 DNA replication origin, thereby arresting viral replication. The first origin (oriL) occurs at 0.4 map units and is in between and immediately adjacent to the HSV-1 DNA polymerase and DNA binding protein genes. The two identical origins of the second type (oriS) occur

1 at 0.82 and 0.97 map units. Numbering below is the terms  
of position relative to the two fold symmetry axis of each  
origin.

5 A. TARGET SITE oriL origin

1. DNA Duplex Target

10 -48 -10  
5' -AGGACAAAGTGCACGCTTCGCGTTCTCACTTTTTT-3'

3' -TTTTTTTCACTCTGCGCTTCGCAAGCGTGAAACAGGA-5'

15 PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

15 5' -TTTTTTGTGTGTTGGGTTGGGTGGGTGTTGTGGT-3'  
(HSVORL1par)

20 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

20 3' -TTTTTTGTGTGTTGGGTTGGGTGGGTGTTGTGGT-5'  
(HSVORL1anti)

25 2. DNA Duplex Target

25 10 47  
5' -AGGACAAAGTGCACGCTTCGCGTTCTCACTTTTTT-3'

30 3' -TCCTCTTCTCGCTTGCAGCGCAAGAGTGAAAAAAA-5'

30 PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

30 3' -TGGTGTGTTGTGGGTTGGGTGGGTGTTGTGGT-5'  
(HSVORL2par)

-58-

## 1 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-TGGTGTGTTGTGGGTTGGGTTGGGTTGTGTGTTTTT-3'  
(HSVORL2anti)5 These two target sites are within the oriL  
origin. Because the oriL also comprises the 5' flanking  
domain of the HSV-1 DNA polymerase and the HSV-1 major DNA  
binding protein, these triplex oligonucleotides may also  
interfere with transcription of those two genes.  
10

## B. TARGET SITE: oriS organ

## DNA Duplex Target

15 -69 -34  
5'-AAGGGGGCGGGGCCGCCTGGTAAAAGAAGTGAGAA-3'  
3'-TTCCCCCGCCCCGGCGGCCATTTCTTCACTCTT-5'

20 PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-TTGGGGGGGGGGGGGGGGGGGGGGTTTTGTTGTGTT-3'  
(HSVORS1par)

25 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-TTGGGGGGGGGGGGGGGGGGGGTTTTGTTGTGTT-5'  
(HSVORS1anti)

30

35

### Example 13

## HUMAN BETA GLOBIN

5 The beta globin gene encodes one of the proteins comprising adult hemoglobin. Mutation in this gene is responsible for beta thalassemia and sickle cell anemia. Triplex oligonucleotides targeted to this gene are designed to inhibit the beta globin gene in thalassemics and in patients with sickle cell anemia, to be replaced by the naturally occurring delta protein. Two classes of triplex oligonucleotides TFO are described, which are targeted against the 5' enhancer or the promotor/coding domain. Numbering is relative to the principal mRNA start site.

#### A. DNA Duplex Target

20 -912 -886  
5'-CCTTTCCCCCTCCTACCCCTACTTCT-3'

3' -GGAAAAGGGGAGGATGGGGATGAAAGA-5'

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

25 3' -GGTTTTGGGGTGGTGGGTTGTTGT-5' (GL1par)

## ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5' -GGTTTTGGGGTGGTTGGGGTTGTTGT-3': (GLlanti)

### B. DNA Duplex Target

-60-

1           5' -AGGAGCAGGGAGGGCAGGAGCCAGGGCTGGGCATAAAAG-3'

3' -TCCTCGTCCCTCCCGTCCTCGGTCCCCACCCGTATTTTC-5'

## 5           PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5' -TGGTGGTGGGTGGGTGGTGGGTGGGTGGGGTTTTTG-3'

(GL2par)

## 10          ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE )

3' -TGGTGGTGGGTGGGTGGTGGGTGGGTGGGGTTTTTG-5'

(GL2anti)

## 15          C. DNA Duplex Target

-36

-9

5' -AGGGCTGGGCATAAAAGTCAGGGCAGAG-3'

20          3' -TCCCGACCCGTATTTCAAGTCCCCTCTC-5'

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5' -TGGGGTGGGGTTTTTGTGTGGGTGTG-3' (GL3par)

## 25          ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3' -TGGGGTGGGGTTTTTGTGTGGGTGTG-5' (GL3anti)

## 30          D. DNA Duplex Target

514

543

5' -CCCTTGATGTTTCTTTCCCTTCTTTCT-3'

-61-

1           3' -GGGAAC TACAAAAGAAAGGGGAAGAAAAGA-5'

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5           3' -GGGTTGTTGTTTGTGTTGGGGTTGTTTGT-5' (GL4par)

## ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

10           5' -GGGTTGTTGTTTGTGTTGGGGTTGTTTGT-3' (GL4anti)

## 10           E. DNA Duplex Target

693

719

15           5' -TTCTTGCTTCCTTTTTTTCTTCTCC-3'

3' -AAGAACGAAAGAAAAAAAAGAAGAGG-5'

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

20           3' -TTGTTGGTTGTTTTTTTTGTTGTGG-5' (GL5par)

## ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

25           5' -TTGTTGGTTGTTTTTTTTGTTGTGG-3' (GL5anti)

## F. DNA Duplex Target

874

900

30           5' -CTCCCTACTTATTTCTTTATTTT-3'

3' -GAGGGATGAAATAAAAGAAAATAAAA-5'

## PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

1           3'-GTGGGTTGTTTTTTTGTTTTTTTT-5' (GL5par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5           5'-GTGGGTTGTTTTTTTGTTTTTTTT-3' (GL6anti)

Example 14

Testing for the effect of oligonucleotide binding  
10         in cells. The effects of triplex-forming oligonucleotides  
        are studied in cell culture. Oligonucleotides are  
        administered to cultured human cell lines, which are then  
        analyzed for oligonucleotide uptake and for a change in  
        the steady-state level of messenger RNA associated with  
15         the DNA target. As an example, the methods for the c-myc  
        gene are shown. One skilled in the art will readily be  
        able to generalize to any gene within a cultured cell.

HeLa cells grown on a solid support (100ul total  
20         volume), are treated with <sup>32</sup>P-labelled oligonucleotide,  
        then incubated as a function of time and concentration.  
        Cells are separated from serum by centrifugation and  
        exhaustive washing, are disrupted by deproteinization then  
        assayed quantitatively on a 8% sequencing gel. This  
25         analysis procedure yields the following characteristics:

a. The apparent partition coefficient for  
        oligonucleotide uptake into HeLa cells.

30         b. The uptake rate, i.e., the time constant to  
        reach a steady state with respect to oligonucleotide  
        uptake.

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1           c.   The half-time for oligonucleotide  
degradation in serum and in the HeLa cell.

5           From those data, the optimized timecourse and  
titration range for the oligonucleotide treatment of cells  
is determined.

10          Transcription inhibition is assayed by a  
variation of the RNase protection assay, which is the  
standard assay for quantifying steady state mRNA levels in  
mammalian cells. Total cellular RNA is extracted from  
oligonucleotide-treated HeLa cells, then hybridized to a  
uniformly labelled antisense RNA transcript, generated by  
the action of T7 polymerase on the SmaI-PvuII human c-myc  
frangment in pSPT19.

20          This SmaI-PvuII probe is complementary to the  
first myc exon and sequences which comprise both the P1  
and P2 transcription start sites of myc. When the probe  
is hybridized in excess over myc transcript, a limit  
RNaseI digest produces either a 0.6 kb duplex  
(transcription from P1, which is the preferred origin in  
HeLa cells) or a 0.4 kb duplex (transcription occurs  
instead from P2, which is used in HeLa cells under  
conditions of serum starvation).

30          The size and quantity of the resulting RNase  
resistant duplexes is then determined by quantitative  
autoradiography on a 5% acrylamide gel matrix. This assay  
system can quantify steady-state RNA levels to within 20%  
accuracy, which is sufficient for the purposes of this  
analysis.

1           The outcome of these cellular titrations is  
analyzed in the context of two control experiments. The  
first is a comparison of the dose response of  
oligonucleotides which bind selectively to the target gene  
5           and the dose response of oligonucleotides which are  
unrelated. If oligonucleotide-mediated repression of the  
c-myc transcription is due to site-specific triplex  
formation in the cell, then an unrelated oligonucleotide  
will not elicit an affect, over an equivalent  
10          concentration range.

The second control addresses the gene specificity  
of the effect. In the RNase protection assay, data are  
always normalized to overall RNA concentration in the  
cell. As such, changes in the steady state level of the  
15          myc transcript are meaningful in their own right. However  
to confirm that the effects of oligonucleotide binding are  
specific to the c-myc gene we also assay for the effect of  
myc-specific oligonucleotide treatment on the steady state  
20          levels of the histone 2A (H2A) message in HeLa cells,  
probing the RNA complement with an H2A antisense RNA,  
generated from a construct which, as for myc sequences,  
has been cloned into a RNA expression vector. When  
25          oligonucleotide mediated repression is specific to the myc  
gene, H2A transcription in HeLa cells will be unaffected,  
over an equivalent concentration range.

Over the 1 to 50 micro-molar range,  
oligonucleotides which bind to the control region of the  
30          human c-myc gene selectively repress c-myc transcription  
in an intact HeLa cell. Preliminary work with other  
oligonucleotides described in the examples have begun to  
display similar selectivity.

1           One skilled in the art will recognize that  
application of these methods is readily generalized to any  
gene in any cell line and is limited only by the  
availability of cloned gene constructs, DNA sequence data,  
5           and a rudimentary understanding of the molecular genetics  
of the gene under investigation. At present, that battery  
of information is available for several hundred human  
genes, and for several thousand genes from other species.

10          The methods can also be applied, without  
significant modification to the use of chemically altered  
oligonucleotides variants, such as those with chemical  
moieties added to the 3' and 5' terminus, oligonucleotides  
with an altered phosphodiester backbone or those with  
15          bases other than G and T (i.e., iodo-G or X).

20          Ultimately, the importance of these examples is  
to show that a whole class of single strand  
oligonucleotide molecules are readily taken up by  
eukaryotic cells, without exogenous manipulation of any  
kind. The uptake mechanism is not known at present, but  
in most cells, it is efficient and, apparently,  
independent of oligonucleotide sequence (Eppstein D.A.,  
Schryver B.B. & Marsh Y.V. (1986) J. Biol.Chem. 261,  
25          5999). Therefore, in the most general sense, the overall  
uptake properties of such oligonucleotides are not  
significantly different from other potent drugs. By this  
criterion, it is certain that an oligonucleotide ligand  
designed to selectively intervene into the process of gene  
30          expression will show pharmacological effects in an intact  
cell.

1        In the past, these cell uptake concepts have been  
1 used to explain the effectiveness of RNA oligonucleotides  
as drugs which enhance the effect of interferon treatment  
(Eppstein D.A., Schryver B.B. & Marsh Y.V. (1986) J.  
5        Biol.Chem. 261, 5999) and of the ability of "antisense" or  
"anti-splice junction" oligonucleotides to selectively  
inhibit mRNA processing in the cell (Heikkile R. et.. al.  
(1987) Nature 328, 445 and Eppstein D.A., Schryver B.B. &  
Marsh Y.V. (1986) J. Biol. Chem. 261, 5999). It is likely  
10      that the same uptake process is the basis for the use of  
triplex-forming oligonucleotides as drugs to selectively  
regulate transcription initiation or to selectively  
destroy a gene target.

15       The design process described herein can be used  
to design a synthetic DNA oligonucleotide which will bind  
specifically to any double strand DNA target of interest.  
The resulting oligonucleotide-duplex DNA complex is best  
described as a colinear triplex. In the triplex the  
20      oligonucleotide molecule occupies the major groove of the  
duplex. The complex is stabilized by base-base hydrogen  
bonding at the surface of the major groove, leaving  
Watson-Crick pairing intact. As a result, the stability  
and site specificity of the synthetic oligonucleotide is  
25      not significantly affected by modification of the  
phosphodiester linkage or by chemical modification of the  
oligonucleotide terminus. Consequently, these  
oligonucleotides can be chemically modified; enhancing the  
overall binding stability, increasing the stability with  
30      respect to chemical degradation, increasing the rate at  
which the oligonucleotides are transported into cells, and  
conferring chemical reactivity to the molecules.

1       Based upon the design method described herein, it  
is possible to design oligonucleotides which are readily  
taken up by eukaryotic cells and, once in the cell, can be  
targeted to specific sites within a genome. Currently,  
5       the site specificity and stability of the synthetic  
oligonucleotide-target site interaction is as good as  
current monoclonal antibody-antigen binding interactions.

10      This new class of site specific molecules can be  
used as gene-specific reagents with the capacity to  
control the transcription process in a gene-specific  
fashion. This control is effective on both somatic genes  
and viral genes which have infected a host cell. When  
15      synthetic oligonucleotides are appropriately coupled to a  
reactive chemical complement, it is possible to create a  
hybrid molecule with the capacity to selectively destroy a  
gene target of interest.

20      One skilled in the art will readily appreciate  
that the present invention is well adapted to carry out  
the objects and attain the ends and advantages mentioned  
as well as those inherent therein. The oligonucleotides,  
compounds, methods, procedures and techniques described  
herein are presently representative of preferred  
25      embodiments, are intended to be exemplary, and are not  
intended as limitations on the scope. Changes therein and  
other uses will occur to those skilled in the art which  
are encompassed within the spirit of the invention or  
defined by the scope of the appended claims.

## 1

## CLAIMS

5        1. A method for making a synthetic oligonucleotide which binds to a target sequence in duplex DNA forming a colinear triplex by binding to the major groove, said method comprising the steps of:

10        scanning genomic duplex DNA and identifying nucleotide target sequences of greater than about 20 nucleotides having either about at least 65% purine bases or about at least 65% pyrimidine bases; and

15        synthesizing said synthetic oligonucleotide complementary to said identified target sequence, said synthetic oligonucleotide having a G when the complementary location in the DNA duplex has a GC base pair, having a T when the complementary location in the DNA duplex has an AT base pair.

20

25        2. The method of Claim 1, wherein said synthetic oligonucleotide is selected from the group consisting of an oligonucleotide oriented 3' to 5' and binding anti-parallel to be about at least 65% purine strand and an oligonucleotide oriented 5' to 3' and binding parallel to the about at least 65% purine strand.

30        3. A synthetic oligonucleotide for forming a colinear triplex with a target sequence in a duplex DNA when said target sequence is either about at least 65% purine bases or about at least 65% pyrimidine bases, comprising,

35

a nucleotide sequence of at least about 20 nucleotides;

1

said nucleotide sequence including G and T,  
wherein G is used when the complementary location  
in the duplex DNA is a GC base pair and T is used  
when the complementary location in the duplex DNA  
is an AT base pair; and

5

said sequence selected from the group  
consisting of an oligonucleotide oriented 3' to  
5' and binding anti-parallel to the about at  
least 65% purine strand in the duplex DNA target  
sequence and an oligonucleotide oriented 5' to 3'  
and binding parallel to the about at least 65%  
purine strand in the duplex DNA target sequence.

10

4. The synthetic oligonucleotide of claim 3,  
wherein, at least one T is replaced with a compound  
selected from the group consisting of X, halogenated  
derivatives of X, I and halogenated derivatives of I.

15

5. The synthetic oligonucleotide of claim 3,  
wherein, at least one G is replaced with a halogenated  
derivative of G.

20

6. The synthetic oligonucleotide of claim 3,  
wherein, at least one base is substituted at the 2'  
furanose position with a non-charged bulky group.

25

7. The synthetic oligonucleotide of claim 6,  
wherein, said non-charged bulky group is selected from the  
group consisting of a branched alkyl, a sugar and a  
branched sugar.

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1           8. The synthetic oligonucleotide of claim 3,  
wherein, the backbone is a phosphodiester analogue which  
is not readily hydrolyzed by cellular nucleases.

5           9. The synthetic oligonucleotide of claim 8,  
wherein, said phosphodiester analogue is selected from the  
group consisting of phosphorothioate, phosphoroselenoate,  
methyl phosphate, phosphoramidite, phosphotriester and the  
alpha enantiomer of naturally occurring phosphodiester.

10          10. The synthetic oligonucleotide of claim 3,  
further including a linker at a terminus.

15          11. The synthetic oligonucleotide of claim 10,  
wherein, said linker is attached to the 3' terminus and is  
selected from the group consisting of a base analogue with  
a primary amine affixed to the base plane through an alkyl  
linkage and a base analogue with a sulfhydryl affixed to  
the base plane through an alkyl linkage.

20          12. The synthetic oligonucleotide of claim 10,  
wherein said linker is attached to the 5' terminus and is  
selected from the group consisting of a base analogue with  
a primary amine affixed to the base plane through an alkyl  
linkage, a base analogue with a sulfhydryl affixed to the  
base plane through an alkyl linkage, a long chain amine  
coupled directly to the 5' hydroxyl group of the  
oligonucleotide and a long chain thiol coupled directly to  
the 5' hydroxyl group of the oligonucleotide.

25          30         13. The synthetic oligonucleotide of claims 10,  
11 or 12 further including a modifying group attached to  
said linker, wherein, said modifying group binds to duplex  
DNA and is selected from the group of molecules consisting

1       of an intercalator, a groove-binding molecule, a cationic  
amine and a cationic polypeptide.

5       14. The synthetic oligonucleotide of claims 10,  
said linker, wherein said modifying group damages DNA and  
is selected from the group of molecules consisting of a  
catalytic oxidant, nitrogen mustard, alkylator,  
photochemical crosslinker, photochemical sensitizer of  
10      singlet oxygen and reagent capable of direct photochemical  
damage.

15      15. The synthetic oligonucleotide of claim 14,  
wherein said photochemical sensitizer of singlet oxygen is  
eosin, methylene blue, acridine orange, or 9 amino  
acridine.

20      16. The synthetic oligonucleotide of claim 14,  
wherein said reagent is ethidium or pyrene derivatives.

25      17. A method of inhibiting the growth of cells,  
comprising the step of administering the synthetic  
oligonucleotide of claim 3 in sufficient amount for  
cellular uptake and binding to the target sequence,  
wherein said target sequence is positioned within the DNA  
25      domain adjacent to the RNA transcription origin.

30      18. The method of claim 17, wherein the cells  
are cancerous cells and the synthetic oligonucleotide is  
specific to the C-myc gene.

1        19. The method of claim 18, wherein the  
synthetic oligonucleotide is selected from the group  
consisting of:

5        3' -TGGTGTGTGGGTTTTGTGGGGGGTGGGGGGTTTTGGGTGGG-5',  
5' -TGGTGTGTGGGTTTTGTGGGGGGTGGGGGGTTTTGGGTGGG-3',  
5' -GTGGTGGGGTGGTTGGGTGGGTGGGTGGGTGGGTGGGT-3',  
3' -GTGGTGGGGTGGTTGGGTGGGTGGGTGGGTGGGTGGGT-5',  
3' -GGTTGGGGTGGGTGGGTGGGTGGGTGGGT-5',  
10      5' -GGTTGGGTGGGTGGGTGGGTGGGTGGGT-3',  
3' -TTTGGTGTGGGGGTGGGGGTTTTGTTTTGT-5',  
5' -TTTGGTGTGGGGGTGGGGGTTTTGTTTTGT-3' and fragments  
and analogues thereof.

15      20. The method of claim 19, wherein, said  
oligonucleotide includes a linker and modifying group.

20      21. A method of inhibiting the growth of pathogens  
comprising the step of administering the synthetic  
oligonucleotide of claim 3 in sufficient amount for cellular  
uptake and binding to the target sequence, wherein said  
sequence binds within the nucleic acid domain adjacent the RNA  
transcription origin.

25      22. The method of claim 21, wherein the pathogen is  
HIV-1 virus and the synthetic oligonucleotide is within the  
viral LTR region.

30      23. The method of claim 22, wherein the synthetic  
oligonucleotide is selected from the group consisting of:

5' -TTTGTTGGGGGTGGGTGGGTGGG-5',  
3' -TTTGTTGGGGGTGGGTGGGTGGG-5',  
5' -TGTGTTGGTTGTGGGGTTTGTGGGTGTGTT-3',

1           3'-TGTGTTGGTTGTTGTGGGGTTTGTGGTGTGTT-5',  
5'-GGGTTGGTGGTGGGGGTGTTGTTG-3',  
3'-GGGTTGGTGGTGGGGGTGTTGTTG-5',  
3'-TGGGTGGGGTGGGTGGGGGTGTGCCCTCTGGG-5',  
5'-GTTTTGGGTGTTGTGGGTGTGTGGTTG-3',  
3'-GTTTTGGGTGTTGTGGGTGTGTGGTT-5',  
5'-TGGGTGGGGTGGGTGGGGGTGTGGGTGTGGGTG-3'

and fragments and analogues thereof.

10          24. The method of claim 23, wherein, said  
oligonucleotide includes a linker and modifying group.

15          25. A method of manipulating the structural  
protein content of epidermal tissue comprising the step of  
administering the synthetic oligonucleotide of claim 3 in  
sufficient amount for cellular uptake and binding to the  
target sequence.

20          26. The method of claim 25 for inhibiting a  
collagen gene, wherein the synthetic oligonucleotide is  
selected from the group consisting of:

25           3'-TGGGTGGGTGGTGGTGGGGGTGTGGTTGTTGGTTT-5',  
3'-GGGTTGGGTGTGGTTGGGGTGGGGTTGG-5',  
3'-GTGGGTGGGTGGTGGTGGGGGTGTGGTTTGG-5' and  
fragments and analogues thereof.

30          27. The method of claim 25, for inhibiting a  
collagenase gene, wherein the synthetic oligonucleotide is  
selected from the group consisting of

5'GGTTGGGGTTGGTGTGTTTTTTGTGTGGGTG-3',  
5'-TTGTGGTTGTTTTTTGGTTGTGTGT-3'  
and fragments and analogues thereof.

1  
5        28. A method of permanently inhibiting gene expression comprising the step of administering the synthetic oligonucleotide of claim 14 in sufficient amount for cellular uptake and binding to the target sequence.

10        29. The method of claim 28, wherein the synthetic oligonucleotide is selected from the group consisting of eosin isothiocyanate, psoralin derivatives, metal chelates, ethidium and pyrene derivatives.

15        30. The method of altering the characteristics of muscle proteins in food animals comprising the step of administering the synthetic oligonucleotide of claim 3 in sufficient amount for cellular uptake and binding to the target sequence.

20        31. The method of claim 30, wherein the synthetic oligonucleotide is selected from the group consisting of:

5' - GTTTTTGGGTGGGGGGGGGGGGGGGG-3' and fragments and analogues thereof.

25        32. A method of inhibiting the interluken 2 alpha chain receptor comprising the step of administering the synthetic oligonucleotide of claim 3 in sufficient amount for cellular uptake and binding to the target sequence.

30        33. The method of claim 32 for inhibiting interluken 2 alpha chain receptor, wherein the synthetic oligonucleotide is selected from the group consisting of:

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1           5'-TTGGGGTGGGGTTTGTGGGTGTGGTTT-3',  
3' TTGGGGTGGGGTTTGTGGGTGTGGTTT-5'  
and fragments and analogues thereof.

5           34. A method of disbursing plaque formation and  
Alzheimer's Disease, comprising the step of administering  
the synthetic oligonucleotide of claim 3 in sufficient  
amount for cellular uptake and binding to the target  
sequence.

10           35. The method of claim 34 for disbursing plaque  
formation in Alzheimer's Disease, wherein the synthetic  
oligonucleotide is selected from the group consisting of:

15           5'-TTTTTGTTTGTYYYYYYYYCCTTCCTTCTTT-3',  
3'-TTTTTGTTTGTYYYYYYYYCCTTCCTTCTTT-5',  
3'-TGGTGGGGGTTGGTGGTTGGTTGGTGT-5',  
5'-TGGTGGGGGTTGGTGGTTGGTTGGTGT-3',  
3'-TTGTGTTTGTGTTGGTGGTTGGGGTGGGGTGGTGTGG-5',  
20           5'-TTGTGTTTGTGTTGGTGGTTGGGGTGGGGTGGTGTGG-3',  
5'-GTGTGTTTGTGTTGGTGGTTGGGGTTTTT-3',  
3'-GTGTGTTTGTGTTGGTGGTTGGGGTTTTT-5',  
3'-GTGTGGTTGGGTGTTGGTGGTGGGTGGGTGTGGT-5',  
5'-GTGTGGTTGGGTGTTGGTGGTGGGTGGGTGTGGT-3',  
25           3'-GGGTGGGTGTGGTGGGGGGTGTGTGGGTGGG-5',  
5'-GGGTGGGTGTGGTGGGGGGTGTGTGGGTGGG-3',  
5'-GGGGTGGGGTGGGGGGGGGGGGGGGG-3',  
3'-GGGGTGGGGTGGGGGGGGGGGGGGGG-5',  
3'-GTGGGGTGGGTGTGTGGGGGGGGGGGGGGGGGG-5',  
30           5'-GTGGGGTGGGTGTGTGGGGGGGGGGGGGGGG-5'  
and fragments and analogues thereof.

1           36. The method of repressing the expression of  
the epidermal growth factor gene comprising the step of  
administering the synthetic oligonucleotide of claim 3 in  
sufficient amount for cellular uptake and binding to the  
5           target sequence.

10          37. The method of claim 36 for repressing  
expression of the epidermal growth factor gene, wherein  
the synthetic oligonucleotids is selected from the group  
consisting of:

15          3' -TGGTGGGGTTGGTGGTTGGTTGGTGT-5',  
5' -TGGTGGGGTTGGTGGTTGGTTGGTGT-3',  
3' -TGGGTGGTGGTGGGGGGGGTGGGTGGG-5',  
5' -TGGGTGGTGGTGGGGGGGGTGGGTGGG-3',  
3' -TTGTGGTGGTGGTGTGGTGGTGGGGTTGGGTGGTGG-5',  
5' -TTGTGGTGGTGGTGTGGTGGTGGGGTTGGGTGGTGG-3',  
3' -TTGTGGTGGGTGGTGGTGGGTGGGTGGTGGTGGTGGTGT-5',  
5' -TTGTGGTGGGTGGTGGTGGGTGGGTGGGTGGTGGTGGTGT-3'  
20          and fragments and analogues thereof.

25          38. A method of repressing the GSTpi gene  
comprising the step of administering the synthetic  
oligonucleotide of claim 3 in sufficient amount for  
cellular uptake and binding to the target sequence.

30          39. The method of claim 38 for repressing the  
GSTpi gene, wherein the synthetic oligonucleotide is  
selected from the group consisting of:

35          5' -GTGTGTGGTGTGGGGGGGGTGGGGGGGGGT-3',  
3' -GTGTGTGGTGTGGGGGGGGTGGGGGGGGGT-5',  
5' -GGGGTGGTGGGTGGTGGTGGTGGTGGTGGTGGTGG-3',  
3' -GGGGTGGTGGGTGGTGGTGGTGGTGGTGGTGG-5',

1                   5'-TTTTTTTTTTTTTTTTTTTTTTTTTT-3',  
1                   3'-TTTTTTTTTTTTTTTTTTTTTTTTT-5'  
and fragments and analogues thereof.

5                  40. A method of intervening into the program of  
cholesterol synthesis by modulating the transcription of  
HMGCoA comprised in the step of administering the  
synthetic oligonucleotide of claim 3 in sufficient amount  
for cellular uptake and binding to the target sequence.

10                 41. The method of claim 40 for modulating the  
transcription of HMGCOA, wherein the synthetic  
oligonucleotide is selected from the group consisting of:

15                 3'-GGTGTGTGTTGGTGGGGTGGGGGTTGTGGGGGG-5',  
5'-GGTGTGTGTTGGTGGGGTGGGGGTTGTGGGGGG-3'  
5'-GGGTGGGTGGTGTGGGGGGTTGTTTGGGGT-3',  
3'-GGGTGGGTGGTGTGGGGGGTTGTTTGGGGT-5',  
3'-TGGGGTTGGGTGGTGGTTGGTTGTTTGGGGGGT-5',  
20                 5'-TGGGGTTGGGTGGTGGTTGGTTGTTTGGGGGGGT-3'

and fragments and analogues thereof.

25                 42. A method of suppression of expression of the  
nerve growth factor receptor comprising the step of  
administering the synthetic oligonucleotide of claim 3 in  
sufficient amount for cellular uptake and binding to the  
target sequence.

30                 43. The method of claim 42 for suppressing the  
gene encoding nerve growth factor receptor, wherein the  
synthetic oligonucleotide is selected from a group  
consisting of:

5'-GGGTTGTGGGTGGTGGGGGGTTGGGTGTGTGG-3',  
3'-GGGTTGTGGGTGGTGGGGGGTTGGGTGTGTGG-5',

1           5'-TGGGGGGGTTGGGTGTGGGTGTTGGGTGTTGG-3',  
3'-TGGGGGGGTTGGGTGTGGGTGTTGGGTGTTGG-5',  
5'-TTGGGTGTTGGGTGGGTGTTGGGTGGGTGGGGTT-3',  
3'-TTGGGTGTTGGGTGGGTGTTGGGTGGGTGGGGTT-5',  
5'-GGGTGGGTTGGGTGTGGGTGGGG-3',  
3'-GGGTGGGTTGGGTGTGGGTGGGG-5',  
5'-GGGGGTGGGGGGGTGGGGGGGGGTGGGGGGGG-3',  
3'-GGGGGTGGGGGGGTGGGGGGGGGTGGGGGGGG-5'.

and fragments and analogues thereof.

10           44. A method for arresting final replication of  
the Herpes Simplex Virus 1 comprising the step of  
administering the synthetic oligonucleotide of claim 3 in  
sufficient amount for cellular update and binding to the  
target sequence.

15           45. A method of claim 44 for arresting viral  
replication, wherein the synthetic oligonucleotide is  
selected from a group consisting of:

20           3'-TTTTGTGTTGGGGGTGGGTGTTGGGGGTGTT-5',  
5'-TTTTGTGTTGGGGGGGTGGGTGTTGGGGGTGTT-3',  
3'-TTTTGTGTTGGGGGGGTGGGTGTTGGGGGTGTT-5',  
5--TTTTGTGTTGGGGGGGTGGGTGTTGGGGGTGTT-3',  
25           5'-TTTTGGGGGGGGGGGGGGGGGTGGGTGTTGGGTGGG-3',  
3'-TTTTGGGGGGGGGGGGGGGGGTGGGTGTTGGGTGGG-5',  
5'-TTTTTTGTGTTGGGTGTTGGGTGGGTGTTGTGGT-3',  
3'-TTTTTTGTGTTGGGTGTTGGGTGGGTGTTGTGGT-5',  
3'-TGGTGTGTTGTGGGTGTTGGGTGGGTGTTGTGTTTTT-5',  
30           5'-TGGTGTGTTGTGGGTGTTGGGTGGGTGTTGTGTTTTT-3',  
5'-TTGGGGGGGGGGGGGGGGGGGGTTTTGTGTGTT-3',  
3'-TTGGGGGGGGGGGGGGGGGGGGTTTTGTGTGTT-5'  
and fragments and analogues thereof.

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1           46. The method of suppressing beta globin genes  
synthesis in thallassemics and sickle cell anemia  
comprising the step of administering the synthetic  
oligonucleotide of claim 3 and sufficient amount for  
5           cellular update combining to the target sequence.

10           47. The method of claim 46 for suppressing the  
synthesis of beta globin gene, wherein the synthetic  
oligonucleotide is selected from the group consisting of:

15           3'-GGTTTGGGGTGGTGGGTTGTTGT-5',  
5'-GGTTTGGGGTGGTGGGTTGTTGT-3',  
5'-TGGTGGTGGGTGGGTGGTGGGTGGGGTGGGGTTTG-3',  
3'-TGGTGGTGGGTGGGTGGTGGGTGGGGTGGGGTTTG-5',  
5'-TGGGGTGGGGTTTTGTGTGGGGTGTG-3',  
3'-TGGGGTGGGGTTTTGTGTGGGGTGTG-5',  
3'-GGGTTGTTGTTGTTGGGGTTGTTGT-5',  
5'-GGGTTGTTGTTGTTGGGGTTGTTGT-3',  
3'-TTGTTGGTTGTTTTTTGGTTGTTGTGG-5',  
20           5'-TTGTTGGTTGTTTTTTGGTTGTTGTGG-3',  
3'-GTGGGTTGTTTTTTGGTTGTTTTTT-5',  
5'-GTGGGTTGTTTTTTGGTTGTTTTTT-3'  
and fragments and analogues thereof.

25

30

35

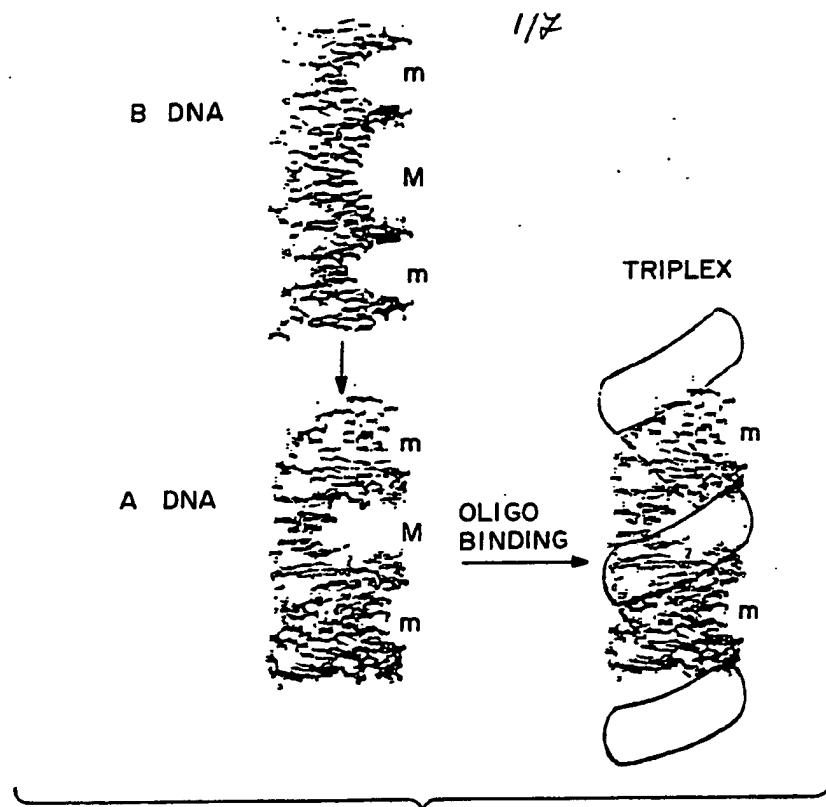


FIG. 1A

**PARALLEL**

5'-----> 3' LIGAND  
 5'-----> 3' ORIENTING STRAND  
 3'-----> 5'

**ANTIPARALLEL**

3'-----> 5' LIGAND  
 5'-----> 3' ORIENTING STRAND  
 3'-----> 5'

FIG. 1B

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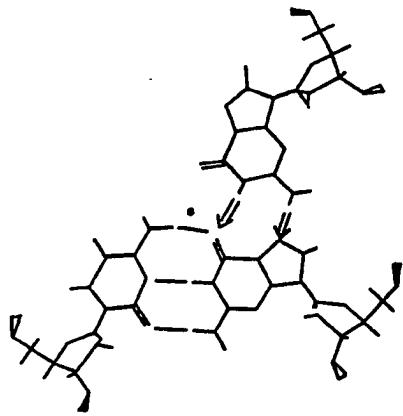


FIG. 2A

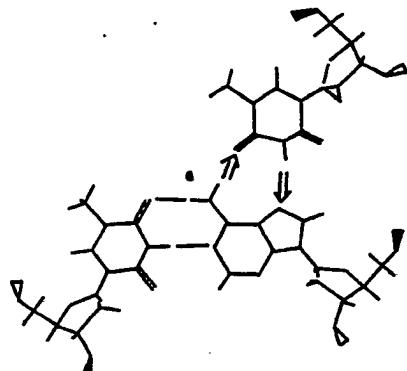


FIG. 2B

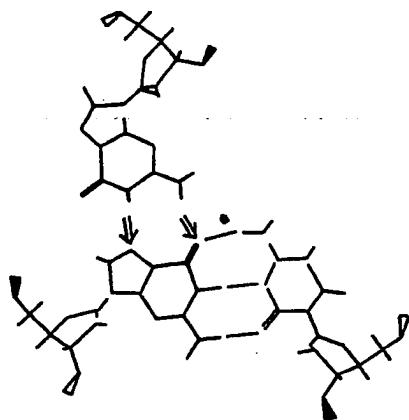


FIG. 2C

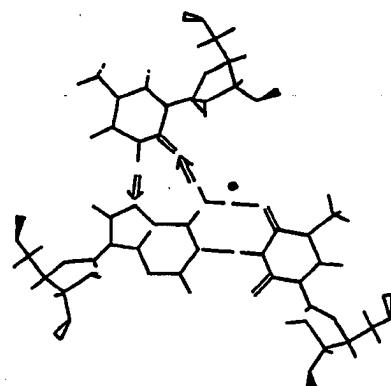


FIG. 2D

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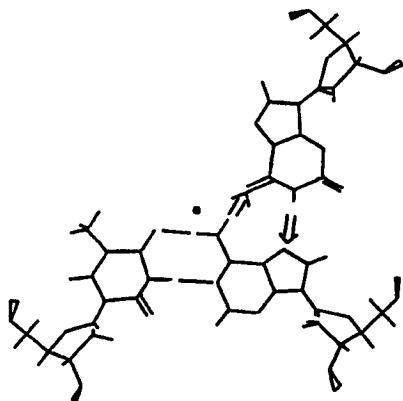


FIG. 3A

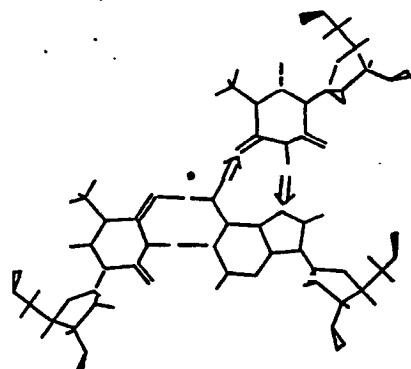


FIG. 3B

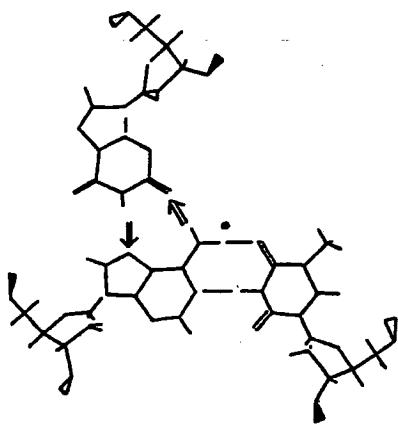


FIG. 3C

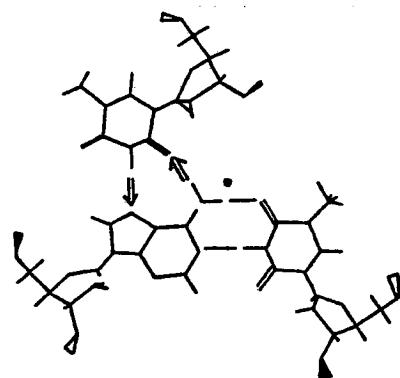


FIG. 3D

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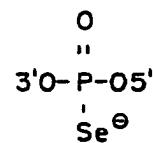
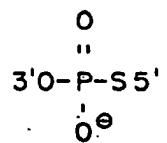
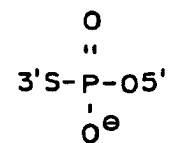
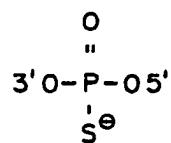


FIG. 4A

FIG. 4B

FIG. 4C

FIG. 4D

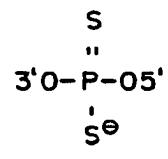
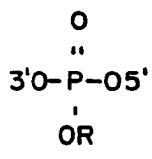
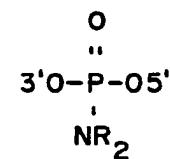
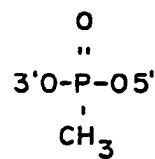


FIG. 4E

FIG. 4F

FIG. 4G

FIG. 4H

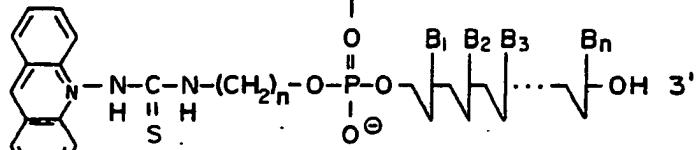
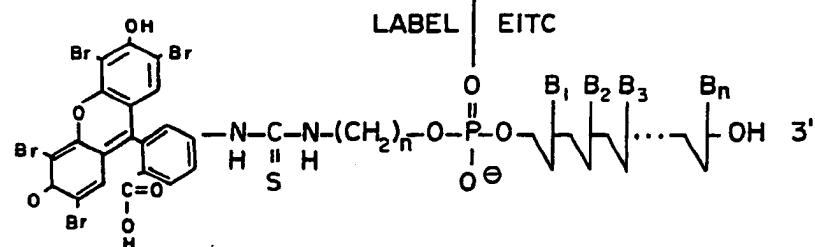
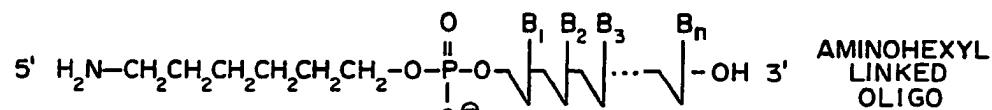


FIG. 5

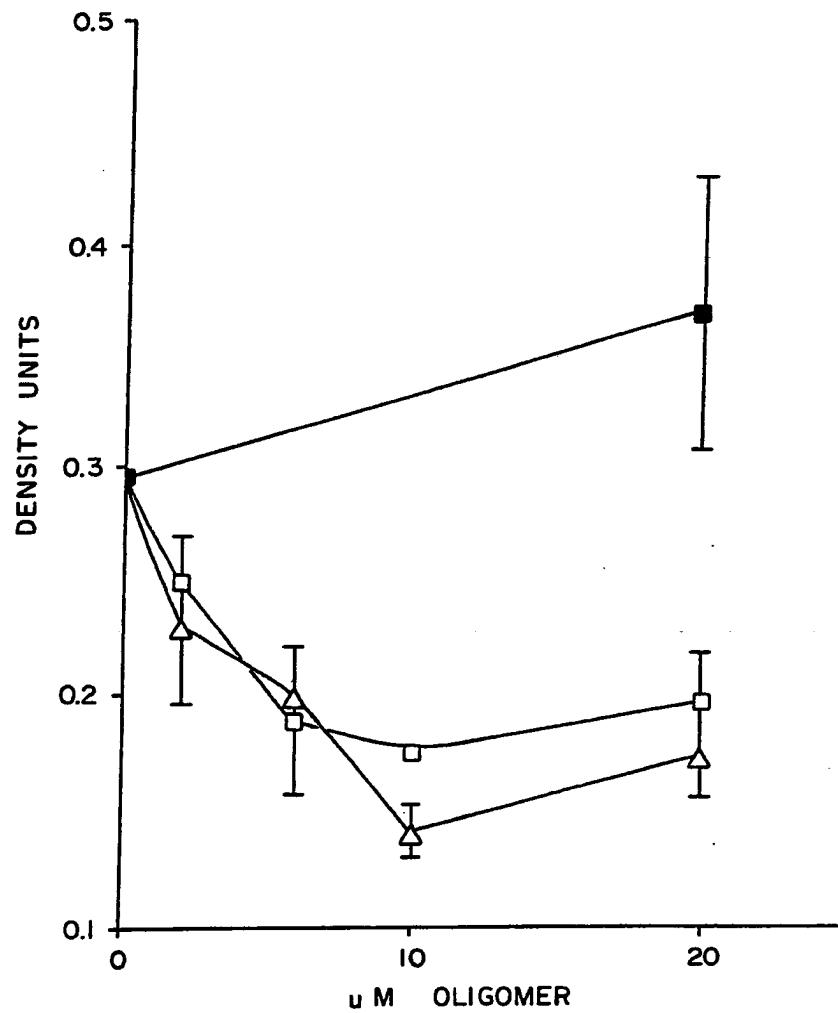
5/ $\lambda$ 

FIG. 6

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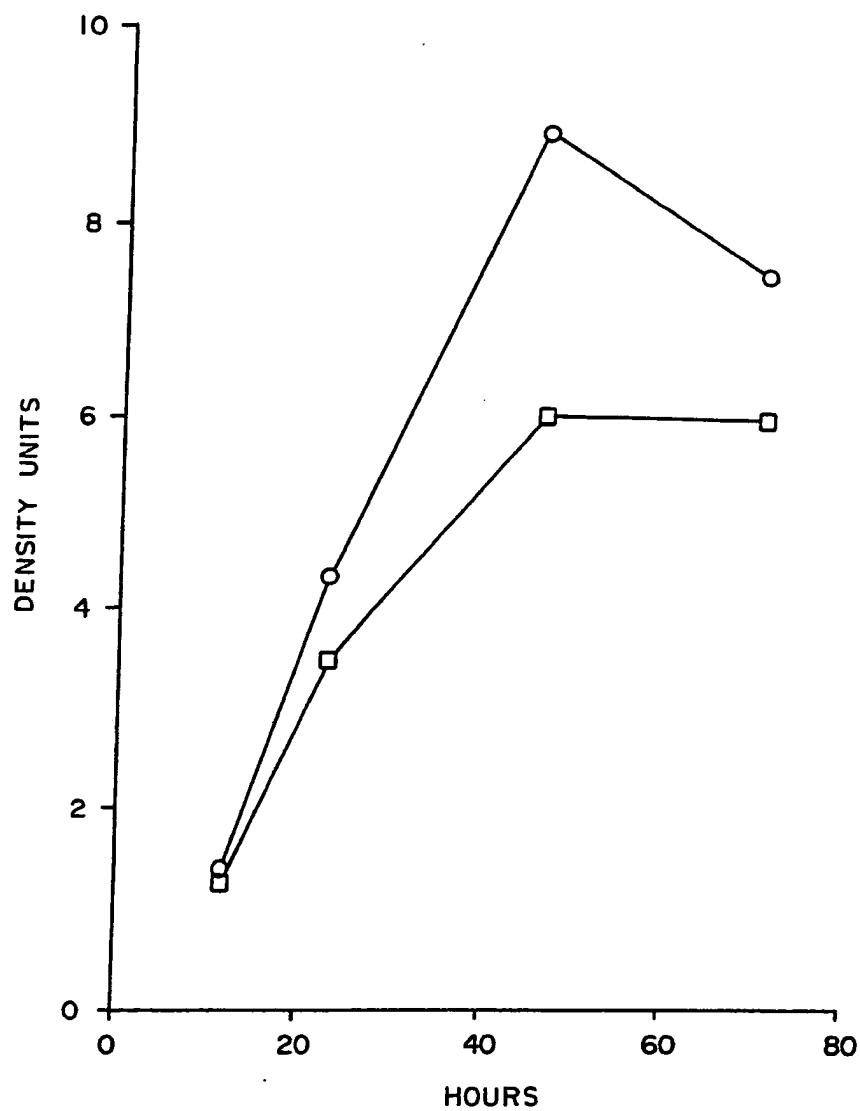


FIG. 7

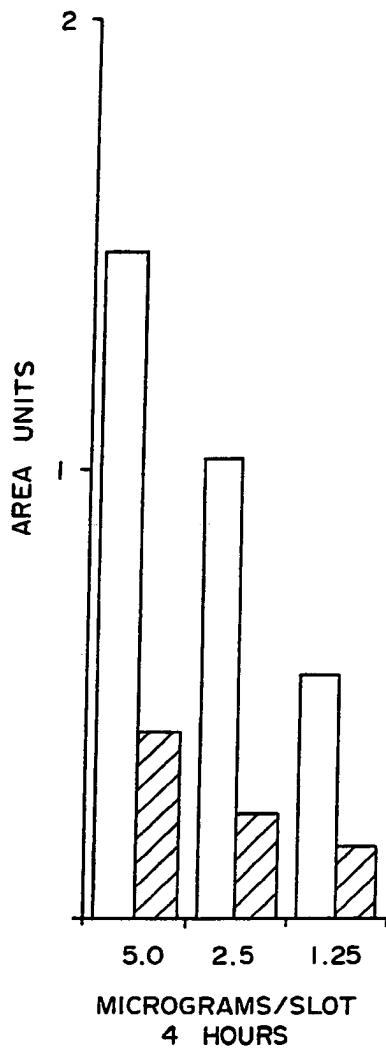


FIG. 8A

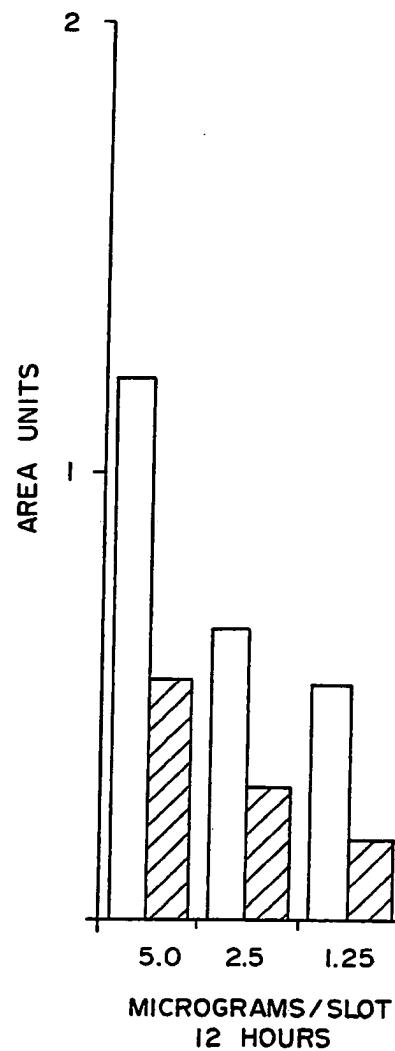


FIG. 8B

SUBSTITUTE SHEET

## INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/05769

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all)	
According to International Patent Classification (IPC) or to both National Classification and IPC	
IPC(5): C07 H 15/12; A22 C 9/00; C12N 15/00; A61 K 48/00	
US: 536/27; 935/33; 424/405; 17/27; 514/44; 514/815; 514/950	
<b>II. FIELDS SEARCHED</b>	
Minimum Documentation Searched <sup>7</sup>	
Classification System	Classification Symbols
US	536/27, 935/33, 514/44, 424/405, 514/80
	514/950, 17/27, 514/815
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>	

CAS, APS, GENBANK, EMBL

### **III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup>**

Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X Y	Journal of Experimental Pathology, volume 2 issued 1985; Minton "The Triple Helix: a Potential mechanism for Gene Regulation" see page 14	3 9
X Y	Physical Chemistry of Nucleic Acids, issued 1974 by Bloomfield et al. Harper & Row Publishers NY NY see pages 322, 323 and 331.	3,4 5,9
X	Nucleic Acids Research, volume 16, number 24, issued June 1988; Francois et al. "Sequence-specific recognition of the major groove of DNA by oligodeoxynucleotides via triple helix formation. Footprinting studies" pages 11431-11440 see abstract.	10, 14
X	Gene, volume 72, issued November 1988, Vlassov et al. "Sequence specific chemical modifications of double stranded DNA with alkylating oligodeoxy-nucleotides derivatives" pages 313-322 see abstract.	3,14

\* Special categories of cited documents:<sup>10</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"S" document member of the same patent family

### **IV. CERTIFICATION**

Date of the Actual Completion of the International Search

Date of Mailing of this International Search Report

25 MARCH 1989

22 MAY 1990

International Searching Authority

Signature of Authorized Officer

ISA/US

  
ROBERT A. WAX

## III DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication where appropriate of the relevant passages	Relevant to Claim No.
X	Proceedings National Academy of Sciences, volume 85, issued March 1988, Praseuth et al. "Sequence-specific binding and crosslinking of and oligodeoxynucleotides to the major groove of DNA via triple helix formation" pages 1349-1353, see abstract.	3,9,14
X	Nucleic Acids Research, volume 16, number 8, issued May 1988 Stein et al. "Physicochemical Properties of Phosphorothionate Oligodeoxynucleotides" pages 3209-3221, see abstract.	3,8,9
X	Proceedings National Academy of Sciences, volume 85, issued 1987, Matsukura et al. "Phosphorothionate analogs of oligonucleotides: Inhibitors of replication and cytopathic effects human immunodeficiency virus" pages 7706-7710, see abstract.	3,8,9,21 22,44,45
X	Proceedings National Academy of Sciences, volume 85, issued July 1988. Walden et al. "Role of RNaseH in hybrid-arrested translation by antisense oligonucleotides" pages 5011-5015, see abstract.	3,46,47
X	Proceedings National Academy of Sciences, volume 85, issued February 1988. Wickstrom et al. "Human promyelocytic leukemia HL-60 cell proliferation and c-myc protein expression are inhibited by an antisense pentadecadeoxynucleotide targeted against cmyc mRNA" pages 1028-1032, see abstract.	3,17,18,19
X	Proceedings National Academy of Sciences, volume 81, issued 1984, Asseline et al. "Nucleic acid-binding molecules with high affinity and base sequence specificity: intercalating agents covalently linked to oligodeoxynucleotides" pages 3297-3301, see abstract.	3,13,14,16

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V.  OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.  Claim numbers \_\_\_\_\_ because they relate to subject matter <sup>12</sup> not required to be searched by this Authority, namely:

2.  Claim numbers \_\_\_\_\_ because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out <sup>13</sup>, specifically:

3.  Claim numbers \_\_\_\_\_ because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI.  OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>14</sup>

This International Searching Authority found multiple inventions in this international application as follows:

See attached sheet

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.  As all searchable claims could be searched without effort ;justifying an additional fee, the International Searching Authority . . . invite payment of any additional fee.

Remark on Protest

The additional search fees were accompanied by applicant's protest.  
 No protest accompanied the payment of additional search fees.

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

- 1) Claims 1-20 entail a method of making an oligonucleotide, an oligonucleotide, and a method of treating cancer, classified in 514/80, 514/44, and 536/27 among others.
- 2) Claims 21-24: antibiotic treatment, classified in 536/27, 935/33, 514/44, and 424/405.
- 3) Claims 25-27: epidermal tissue treatment, classified in 514/44 and 514/950.
- 4) Claims 28-29: gene treatment, classified in 935/33 and 514/44.
- 5) Claims 30-31: meat treatment aids, classified in 17/25, 935/33 and 514/44.
- 6) Claims 32-33: interluken receptor modification, classified in 935/33 and 514/44.
- 7) Claims 34-35: method of treating Alzheimers disease, classified in 935/33 and 514/44.
- 8) Claims 36-37: method of affecting the expression of epidermal growth factor, classified in 935/33 and 514/44.
- 9) Claims 38-39: method of repressing the function of GSTpi gene, classified in 935/33 and 514/44.
- 10) Claims 40-41: method of affecting cholesterol synthesis, classified in 935/33 and 514/44.
- 11) Claims 42-43: method of affecting nerve growth, classified in 935/33 and 514/44.
- 12) Claims 44-45: antiviral agents, classified in 935/33 and 514/44.
- 13) Claims 46-47: globin affecting agents, classified in 514/815, 935/33 and 514/44.

All of these inventions pertain to a different invention. Each of the different inventions forms a separate inventive identity and requires a different search of the patent classes as well as a separate search of the Genbank and EMBL databases.

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